DISSERTATION

DROUGHT STRESS AND RECOVERY IN GREEN ASH (*Fraxinus pennsylvanica* Marsh.)

Submitted by
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In partial fulfillment of the requirements
For the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Summer 2009

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY GREGORY LITUS ENTITLED DROUGHT STRESS AND RECOVERY IN GREEN ASH (*Fraxinus pennsylvanica* Marsh.) BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF THESIS
DROUGHT STRESS AND RECOVERY IN GREEN ASH
(*Fraxinus pennsylvanica* Marsh.)

Throughout the Front Range of Colorado, municipalities have developed urban forest management plans that focus on preserving the health of landscape trees and promoting an increase in the canopy cover as an offset to carbon dioxide emissions through carbon sequestration. However, drought in recent years has prompted a concerted effort to conserve water used for landscape irrigation. The combined drought and reductions in irrigation have the potentials to increase water stress in shade trees and lessen the amount of carbon sequestered. To assess the effects of drought stress on growth, photosynthesis and long term health of established green ash (*Fraxinus pennsylvanica* Marsh.), a record dry 2005-2006 winter was exploited so that severe drought stress could be induced. Early season drought reduced spring leaf growth by 25 percent compared to controls. As drought progressed through the growing season, the stressed trees increased intrinsic water use efficiency by controlling stomatal conductance, based on threshold water potentials, while maintaining photosynthesis. After irrigation was applied in late summer, tree water potentials, stomatal conductance and photosynthesis recovered to near pre-drought levels. The decreased photosynthesis contributed to the reduction in tree growth for the season but did not alter total non-structural carbohydrates concentrations or produce a carbohydrate deficit that would dramatically hinder growth in subsequent years. The extended drought stress followed by irrigation did not affect dormancy and cold hardiness was maintained to -50 ^{o}C. Potted green ash trees were used to determine the extent of drought stress tolerated by green ash. At a predawn leaf water

potential of -5.28 MPa, stomatal conductance and photosynthesis were reduced but still measureable. Established trees exposed to severe drought conditions did not experience predawn leaf water potentials below -3.14 MPa. Considering the range of drought stress tolerated by green ash and the unlikelihood of those conditions occurring in a managed landscape, negative effects of drought stress are minimized as long as late season irrigation can be applied. However, in green ash, the timing of drought stress can permanently restrict growth in any single year and significantly reduce the total carbon sequestered through photosynthesis.

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Thanks Pop

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Symbols and Acronyms

ARDEC ..... Agricultural Research Development and Education Center
A_n ..... net assimilation (μmol $m^{-2}s^{-1}$)
C_t ..... specific conductance (μScm^{-1} at time t)
Ctrl ..... control
ET ..... evapotranspiration
ET_o ..... reference evapotranspiration
g_s ..... stomatal conductance (mmol $m^{-2}s^{-1}$)
G_s ..... canopy conductance (mmol $m^{-2}s^{-1}$)
ModR ..... moderate drought stress with recovery irrigation
ModNR ..... moderate drought stress without recovery irrigation
PAR..... photosynthetically active radiation (μmol $m^{-2}s^{-1}$)
PIP..... pot in pot
Ψ_{pd} ..... predawn leaf water potential (MPa)
Ψ_l ..... leaf water potential (MPa)
RWC ..... relative water content (%)
SevR..... severe drought stress with recovery irrigation
σ..... membrane leakage ($\mu Scm^{-1}mg^{-1}$)
σ_{max}..... maximum membrane leakage ($\mu Scm^{-1}mg^{-1}$)
σ_{rel}..... percent maximum leakage (%)
TNC..... total non-structural carbohydrates
VPD ..... vapor pressure deficit (kPa)

Chapter 1 Growth and Photosynthesis

1.1) Introduction and Literature Review

This dissertation reports on the relevant aspects of research conducted on established male green ash (*Fraxinus pennsylvanica* Marsh. 'Patmore') trees from 2005 to 2006 at the Agricultural Research Development and Education Center (ARDEC).

Research on the physiology of plant drought stress is extensive and much of the physical and biochemically controlled responses to drought stress have been thoroughly described for economically important plants (Aphalo and Jarvis, 1993b; Chaves, 1991; Comstock, 2002; Hsiao, 2000; Jones, 1998; Niinemets et al., 2004; Passioura and Munns, 2000; Schymanski et al., 2007; Sperry et al., 2002; Tyree and Sperry, 1988). More specifically, the immediate effects of drought stress on cell turgor and consequent shoot elongation and leaf expansion is well documented (Frensch, 1997, Hsiao, 2000, Lovisolo et al., 2002, Marron et al., 2003, Metcalfe et al., 1990, Passioura and Munns 2000). Likewise, reduction of photosynthesis as a result of drought stress has been intensely researched in many species other than green ash. Studies of leaf water potentials (Ψ_l), stomatal conductance (g_s) and photosynthesis, more accurately termed net CO_2 assimilation (A_n), in a diversity of hardwood tree species are extensive (Arndt et al., 2001; Angelopoulos et al., 1996; Aspelmeier and Leuschner, 2004; Behboudian et al., 1986; Bovard et al., 2005; Cochard et al., 2002; DeLucia and Schlesinger, 1991; Hogg et al., 2000; Oren and Pataki, 2001; Wullschleger et al., 2000). To better understand the potential response of green ash trees to drought stress, it is important to look at studies specific to green ash, different species within the genus such as white ash (*Fraxinus americana* Marsh) and European

ash (*Fraxinus excelsior* L.), and species within the same family (Oleraceae), such as olive (*Olea europea* L.). Abrams et al. (1990) and Shumway et al. (1991) both used green ash as a model to look at variations in plant phenology across a longitudinal gradient from New York to South Dakota. Abrams looked at changes in leaf morphology across this gradient to see if the increasingly dry climate (east to west) resulted in phenotypic changes and produced genotypes that supported drought resistance. Although phenotypic variation in leaf morphology did not directly apply to my study, the measurements of Ψ_l and leaf gas exchange from Abrams and Shumway provided insight into the range of results expected in green ash. Abrams found a significant linear relationship between Ψ_l and A_n. At Ψ_l greater than –1.0 MPa, A_n was as high as 7.5 umol $m^{-2}s^{-1}$. During the end of the simulated drought, mid-day Ψ_l reached –3.5 MPa and leaves still maintained A_n between 1.5 and 3.0 umol $m^{-2}s^{-1}$. Shumway evaluated xylem architecture in seedlings and found it to be essentially fixed by mid-summer with leaf area of the seedlings increasing by 37 percent in well watered trees having predawn leaf water potentials (Ψ_{pd}) greater than -0.1 MPa. Drought stressed trees ($\Psi_{pd} > -0.6$ MPa) showed no such growth in leaf area. This indicated that even minor levels of drought stress reduced leaf growth. Shumway concluded that whole plant responses to drought involve coordinated changes in many variables.

Research utilizing "Marshall's Seedless" green ash in an urban setting, Whitlow et al. (1992) found that established and potted well-watered ash achieved a maximum g_s of 10.5 mmol $m^{-2}s^{-1}$. The range of g_s was quite variable and did not correlate with Ψ_l as low as –2.3 MPa. Ψ_{pd} was higher than –1.0 MPa throughout the duration of the study.

Whitlow's research suggests that street trees in New York City were not subject to unusually high levels of drought stress.

Findley (1999) worked on young green ash at ARDEC to study the impact of irrigation regimes on growth, fall color and cold hardiness. His results showed that on September 3, 1998, at the peak of drought stress, excessively irrigated trees had similar Ψ_{pd} to trees receiving half as much irrigation. Only trees receiving 25 percent as much irrigation had Ψ_{pd} significantly lower than the two treatments receiving greater irrigation. However, even the treatment receiving the least water had Ψ_{pd} greater than –0.9 MPa. The high Ψ_{pd} was likely due to a reservoir of stored soil moisture that requires extended periods of drought to deplete. Findley found that shoot elongation and leaf expansion were complete by the beginning of irrigation, on June 1, 1998. Irrigation treatments, applied after June 1, 1998, were insufficient to produce statistically significant differences in growth. Any reduction in growth would have required longer drought stress that was able to alter winter soil water storage between the treatments. Although Findley's work has the most direct application to my research, the results have limited utility because only mild drought stress was achieved and more detailed measurements of g_s and A_n were not collected.

Pataki and Oren (2003) found that white ash canopy conductance (G_s) followed an expected diurnal trend with increasing G_s correlated with increasing vapor pressure deficit (VPD) and net radiation. Leaf specific g_s varied from 150-600 mmol $m^{-2}s^{-1}$ in the sun to 50-150 mmol $m^{-2}s^{-1}$ in the shade. Reductions in soil moisture did not initiate

responses in white ash but the drop in soil moisture may not have been sufficient to trigger a stress response. Joyce and Steiner (1995) also researched the variability of white ash xylem conductance. They confirmed that apical dominance affects xylem growth and subsequent leaf g_s. The same applied to south facing sun leaves and north facing shade leaves. The shoots on the south side of the trees had a higher leaf g_s supporting greater evapotranspiration. My research looked at each study tree as a unit with the functional balance, which controls leaf development, explaining much of the variability between individual leaf measurements.

Carlier et al. (1992) measured stomatal conductance and water potentials in a native stand of European ash. They measured Ψ_{pd} as low as –4.8 MPa with minimum Ψ_l as low as –5.5 MPa. Maximum g_s was 200 mmol $m^{-2}s^{-1}$ for well hydrated trees. Stomata partially closed at noon in well hydrated trees and as early as 07:00 in drought stressed trees. Hölsher (2004) measured maximum A_n values in the upper canopy of European ash at 16.3 μmol $m^{-2}s^{-1}$. In the lower canopy, with less irradiance, max A_n was 12.0 μmol $m^{-2}s^{-1}$ and in saplings A_n ranged between 5.0 and 6.4 μmol $m^{-2}s^{-1}$. In European ash, grown in full sunlight at ambient CO_2 without drought stress, Keiller and Holmes (2001) measured g_s at 155 mmol $m^{-2}s^{-1}$ and A_n at 10.8 umol $m^{-2}s^{-1}$. Measuring stem flow in European ash saplings growing on shallow drought susceptible soils in western Germany, Stöhr and Lösch (2004) found that maximum daily sap flow rates occurred shortly after sunrise and declined continuously during the day in drought stressed trees. After recovery from drought stress, persistently reduced hydraulic conductance was due to continued stomatal closure that was correlated with duration of drought.

In olive, Dichio et al. (2005) found a high degree of leaf morphologic phenotypic plasticity during periods of drought, and rapid recovery of photosynthesis upon watering. Like white and European ash, olive, stressed to Ψ_{pd} of –5.35 MPa, had dramatically reduced g_s and A_n that were less than 5 percent of control during mid-day. However, a minimal level of A_n was maintained. After 29 days of recovery from drought, osmotic potentials increased but remained lower than measured values prior to drought. Angelopoulos et al. (1996) found that maximum A_n for olive was around 08:00 with daily stomatal depression occurring over the next few hours and minimum A_n in drought stressed plants occurring around 11:00. The most severely stressed plants reached Ψ_{pd} of –5.7 MPa. At that level, olive, a sclerophyllous drought-tolerant plant, was able to maintain A_n at about 30 percent of well hydrated control plants.

The research cited suggested that the green ash at ARDEC would be able to withstand dramatic levels of drought stress with Ψ_{pd} of –5.0 MPa or lower while still maintaining a minimal level of A_n. Diurnal g_s should peak sometime in the morning and begin to decline through the day as VPD increased. A_n should parallel this trend since it is highly correlated with g_s. Drought stressed trees should see a substantial reduction in both g_s and A_n that would also follow a diurnal trend associated with VPD. Low soil water potentials would reduce both parameters even further. Upon watering, both g_s and A_n values should rebound to levels slightly lower than levels measured prior to stress and remain there as long as additional drought stress is avoided.

Chapter 1 provides the results of tests to the hypothesis that green ash will withstand declining Ψ_{pd} and, upon watering, recover from depressed g_s and A_n during periods of drought.

1.2) Methods

Site Characteristics

In 1996, the Tree and Turf Research Area was established at ARDEC, located approximately 16 kilometers northeast of Fort Collins, Colorado. Each of nine replicated blocks were divided into three subunits planted with either; 1) male green ash trees with a bluegrass (*Poa pratensis* L. ssp *pratensis* 'Livingston') understory, 2) male honeylocust (*Gleditisia triacanthos* L. var *inermis* (L.) Schneid 'Skyline') trees with a blue grass understory, or 3) bluegrass turf only (Figure 1.1). Each subunit was planted with three rows of nine trees on 3.7 meter centers aligned in an east/west direction. Each row was spaced 4.9 meters apart. Overhead sprinkler irrigation was installed such that each of the nine blocks could be independently irrigated. Irrigation was used to augment the average yearly precipitation of 384 mm (Western Regional Climate Center). Nine nuclear density gauge access tubes were installed in each block to a depth of 1.5 meters. The site is surrounded by a cottonwood (*Populus deltoides* Bartram ex Marsh.) windbreak to the west and northeast, elms (*Ulumus americana* L.) to the north and Austrian pine (*Pinus nigra* Arnold) to the south. A gap in the windbreaks to the east allows uninterrupted summer winds from the east and southeast. The soil is a Fort Collins clay (fine, mixed, mesic Ustollic Haplargid). Prior to establishment of the site, Blocks 2 through 9 had a pH between 8.0 and 8.2, organic matter ranged from 1.3 to 1.8 percent

and nutrients concentrations were uniform across the site. Block 1 had lower pH (7.7) and higher concentrations of nitrogen, potassium, zinc, iron and manganese.

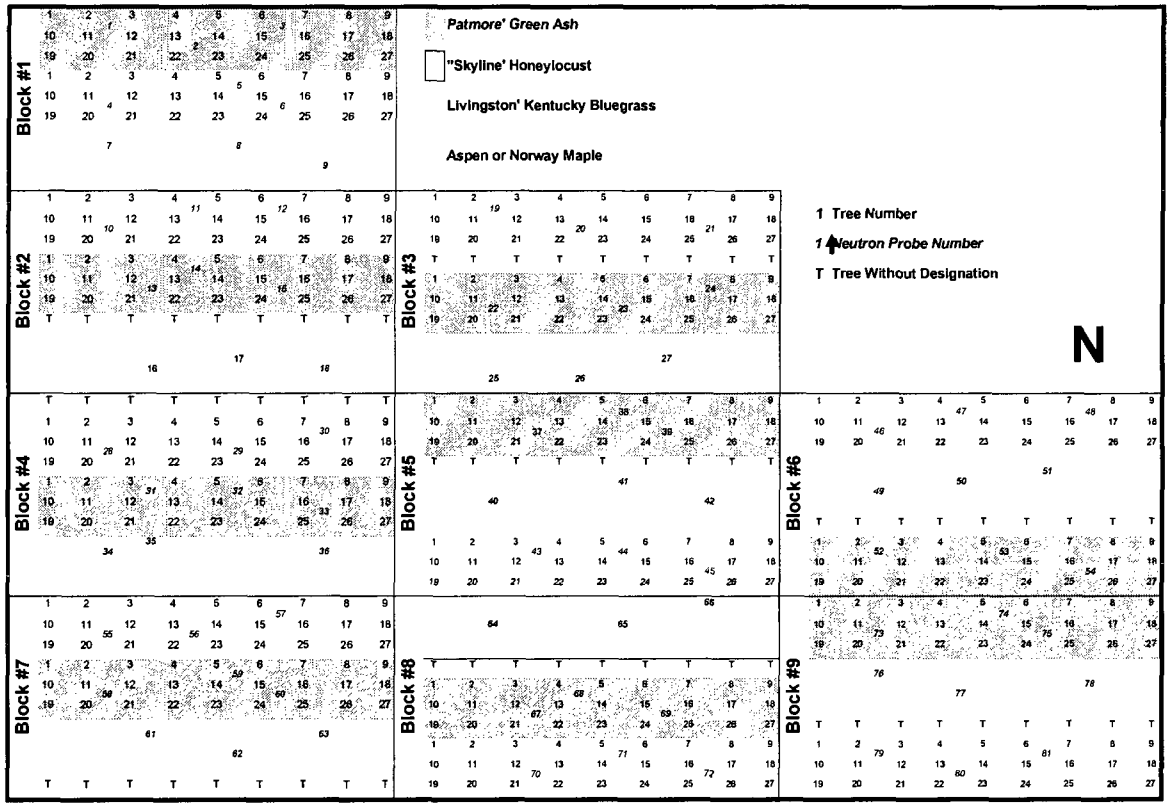


Figure 1.1: Diagram of the Agricultural Research Development and Education Center (ARDEC) Tree and Turf Research Site. Measurements in this study were collected on the green ash (gray highlight) in all blocks except Blocks 6 and 8. Block 1 in the northwest corner of the research site was used as a control with irrigation throughout the growing season. Windbreaks to the north, south, west and northeast are not shown.

Block 1 represented a unique block due to a secondary water source that likely comes from leakage of an adjacent irrigation holding pond. The green ash trees in Block 1 were significantly larger than all other blocks in height, canopy size and trunk diameter (Appendix A). Block 1 was thus used as a control block (Ctrl) with trees grown under fully hydrated conditions. The remaining eight blocks were originally established to measure the effect of differing irrigation treatments but, in 2005, mean trunk diameters

for the seven interior trees in each block were not significantly different (Appendix A). The irrigation treatments were maintained during 2005. However, in 2006, after confirmation that past irrigation did not significantly affect treatment blocks, Blocks 2 through 9 were considered equivalent.

The research presented in this dissertation was conducted in 2005 and 2006 on the green ash sub-blocks. During 2005 drought stress and associated measurements were limited to Blocks 1, 2, 4, and 5. In 2006 Blocks 1, 2, 3, 4, 5, 7, and 9 were used. Block 6 was excluded due to anomalous soil moisture measurements that may be due to a variation in soil type. Block 8 was excluded due to an early season irrigation failure that flooded land bordering the green ash stand.

Between November 1, 2005 and May 1, 2006 the site received only 50 mm of precipitation (Table 1.1), the lowest on record, and that lack of precipitation resulted in less than a 4 percent volumetric moisture increase to the upper 1.5 meters of soil. The severe depletion of soil moisture provided the perfect opportunity to assess the response of established green ash to sustained drought.

	Historical Monthly Average (mm) 1971-2000	ARDEC (mm) 2005	2006
Jan	10.2	-	2.5
Feb	11.7	-	9.4
Mar	28.4	-	23.6
Apr	55.1	-	9.4
May	86.6	-	20.1
June	55.9	-	3.8
July	38.1	-	19.8
Aug	40.4	-	11.2
Sept	40.9	-	21.1
Oct	32.8	69.6	-
Nov	14.0	3.6	-
Dec	6.4	2.0	-

Table 1.1: Historical monthly average and actual precipitation for the ARDEC research site from October 2005 through September 2006. Precipitation for ARDEC was collected by the Western Regional Climate Center (WRCC) at a meteorological station located approximately 3 kilometers south of the site.

2005 Irrigation

The objective of experiments in 2005 was to maintain historic irrigation treatments for the majority of the site while conducting a limited drying of Blocks 4 and 5. Beginning in June of 2005, prior to drying experiments, a strict protocol of irrigation was established that reflected the original treatment design. Blocks 1, 4 and 8 received 160 percent of calculated bluegrass ET (ET_o). Blocks 3, 6, and 7 received 80 percent of ET_o. Blocks 2, 5 and 7 received 40 percent of ET_o (Figure 1.1). Irrigation was applied when the ET_o deficit reached 25 mm, as calculated by the Northern Colorado Water Conservation District (NCWCD) based on Allen et al. (1998), using weather data collected at the CSU Horticulture Research Center located approximately 3 kilometers south of the ARDEC research site. NCWCD used a standard quality turf adjustment that reduced ET_o by 0.03 to 0.05 mm and represented a slower growing, less dense and lighter color turf exhibiting minor signs of drought stress.

To simulate drought, Block 4 was not irrigated from July 17, 2005 until September 2, 2005 when approximately 50 mm of water was applied. Similarly, Block 5 was not irrigated from July 17, 2005 until August 28, 2005 when approximately 50 mm of water was applied.

2005 Tree and Soil Measurements

Soil moisture measurements were obtained using a Troxler Model 4300 nuclear density soil moisture gauge at point specific depths corresponding to 0.3, 0.6, 0.9 and 1.22 meters below ground surface. Measurements were taken in all 81 site-wide access tubes, or in block specific subsets of the tubes, from June 18, 2005 through September 14, 2005 on dates corresponding to water potential measurements and transpiration monitoring requirements (Table 1.2).

Leaf transpiration was measured using a LiCor Li-1600 porometer on dates chosen to bracket the effects of drought stress and recovery irrigation. On dates shown in Table 1.2, between three and five leaves were measured from each designated trees such that each tree was visited multiple times during the measurement period. On August 24, 2005 a complete set of measurements detailing diurnal change were collected from Block 1and Block 5. These blocks represented the end points of the fully hydrated control (Block 1) and drought stressed treatment (Block 5). Ψ_{pd} and Ψ_l were measured using a pressure chamber. Ψ_{pd} was an average of a single leaf collected from the lower canopy of three trees in each block. When possible, Ψ_l was measured from leaves also measured for transpiration.

The effects of soil moisture and VPD on Ψ_{pd} were analyzed using a general linear regression model (SAS Institute, 2008) at the 0.05 significance level. Differences between means of gs and Ψ_{pd} were tested using Student T-tests at the 0.05 significance level.

Date	Soil Moisture	Predawn Leaf Water Potential	Mid-day Leaf Water Potential	Porometer	
				Block	Time
Jun 18	Site wide				
Jul 06	Site wide				
Jul 08		1,2,4,5			
Jul 28	Site wide		1,2,4,5		
Jul 30		1,2,4,5			
Aug 02	1,2,4,5	1,2,4,5			
Aug 03	1,2,4,5	1,2,4,5			
Aug 04	1,2,4,5				
Aug 05		1,2,4,5			
Aug 06				4	15:30-16:30
Aug 07				4	10:00-11:45
Aug 08	1,2,4,5	1,2,4,5			
Aug 09				1,2,4,5	09:38-10:30
Aug 16			1,2,4,5	1,2,4,5	10:17-11:41
Aug 17	Site wide	1,2,4,5			
Aug 22		1,2,4,5			
Aug 24		1,2,4,5	1,5	1,5	08:38-20:45
Aug 27	1,2,4,5	1,2,4,5	1,4,5	1,4,5	08:07-19:47
Aug 28		1,2,4,5			
Aug 31	Site wide	1,2,4,5	1,2,4,5	1,2,4,5	11:05-14:29
Sep 02	1,2,4,5	1,2,4,5			
Sep 03	1,2,4,5	1,2,4,5	1,2,4,5	1,2,4,5	12:14-14:29
Sep 04	1,2,4,5	1,2,4,5		1,2,4,5	13:02-14:15
Sep 05	1,2,4,5	1,2,4,5			
Sep 06	1,2,4,5	1,2,4,5			
Sep 07	1,2,4,5	1,2,4,5	1,2,4,5		
Sep 07	2				
Sep 08	1,2,4,5	1,2,4,5			
Sep 14	Site wide				

Table 1.2 2005 data collection schedule. Site wide soil moisture measurements included nuclear density gauge access tubes in all 9 Blocks. Ψ_{pd} was collected from trees: 1F, 1G and 1H (Control Block 1), 2D, 2E, and 2F (40% ET_o irrigation Block 2), 4F, 4G, and 4H (drought stressed Block 4), 5B, 5C, and 5D (drought stressed Block 5). Mid-day Ψ_l and porometer measurements were collected from a subset of the trees used for Ψ_{pd}.

2006 Irrigation

The intent of this 2006 irrigation schedule was to assess the recovery of green ash as a function of progressively longer seasonal drought. Beginning on May 1, 2006, Block 1 was maintained as a fully hydrated control and irrigated at 160 percent of calculated ET_o until September 30, 2006 when the entire site was irrigated with approximately 75 mm of water over a period of 4 days.

Specifically for 2006, all treatment blocks 2, 3, 4, 5, 7, and 9 were not irrigated until a predetermined Ψ_{pd}, measured as the average value for the seven interior trees, was achieved (Table 1.3). When that prescribed stress level was reached in a specific treatment block, a one-time irrigation of either 26 or 51 mm of water was applied over an 8 hour evening period. Irrigation dates for the stress blocks were: Block 9 – August 8, 2006 (26 mm), Blocks 2 and 5 – August 23, 2006 (51 mm), and Blocks 3 and 4 – August 29, 2006 (51 mm). Block 7 did not reach the prescribed Ψ_{pd} and was not irrigated until the site-wide irrigation on September 30, 2006. The six treatment blocks and one control block were given designations based on the level of stress received (Table 1.3). Block 1 was a fully hydrated control (Ctrl) with Ψ_{pd} greater than -0.80 MPa. Blocks 2, 3, 4 and 5 received severe drought stress and recovery irrigation (SevR) with Ψ_{pd} ranging between -1.48 and -3.00 MPa. Block 7 received moderate drought stress and no recovery irrigation (ModNR) with Ψ_{pd} ranging between -0.83 and -2.14 MPa. Block 9 received moderate drought stress and recovery irrigation (ModR) with Ψ_{pd} ranging between -1.10 and -1.97 MPa.

Stress Designation	Abbreviation	Block	Minimum Ψ_{pd} Reached Block Average (MPa)	Means Separation	Date
Irrigated Control	Ctrl	1	-0.72 ± 0.07	A	September 04, 2006
Severe with recovery irrigation	SevR	2	-2.37 ± 0.49	BC	August 23, 2006
		3	-2.43 ± 0.35	B	August 24, 2006
		4	-2.33 ± 0.49	BC	August 24, 2006
		5	-1.92 ± 0.52	DC	August 23, 2006
Moderate without recovery irrigation	ModNR	7	-1.36 ± 0.43	E	August 23, 2006
Moderate with recovery irrigation	ModR	9	-1.78 ± 0.30	DE	August 06, 2006

Table 1.3 Specific drought treatments as defined by Ψ_{pd} and irrigation of seven green ash treatment blocks in 2006. The designation Ctrl is used for the control Block 1 that received irrigation at the rate of 160 percent of bluegrass ET for the duration of the study. The designation SevR applies to Blocks 2, 3, 4 and 5 that were subjected to severe drought until the initiation of recovery irrigation on Block specific dates in late August, 2006. The designation ModNR applies to Block 7 which was subjected to moderate drought but did not receive recovery irrigation until September 30, 2006. The designation ModR applies to Block 9 which was subjected to moderate drought and then irrigated on August 8, 2006. Block averages represent the date with the lowest mean Ψ_{pd} of seven interior trees in each drought stressed block and three interior trees in Block 1. For means separation blocks with the same letter are statistically equivalent at a 0.05 significance level. Error term with block average is one standard deviation.

2006 Tree and Soil Measurements

Soil moisture measurements were collected using the same Troxler Model 4300 nuclear density soil moisture gauge used in 2005. In addition to the soil depths measured in 2005, a measurement at 1.52 meters below ground surface was added. Stomatal conductance and A_n were measured using a PP Systems Cirus II gas exchange analyzer set with a constant ambient CO_2 of 380 ppm.

The different treatment blocks were monitored for g_s and A_n at a frequency sufficient to assess the changing effects of extended drought and focused on the increased stress associated with high VPD and PAR at mid-day (Table 1.4). Diurnal measurements were collected on selected days to assess the changes in gas exchange with environmental changes as the day progressed. Prior to and after recovery irrigation, monitoring was

increased to include seven trees within the targeted treatment block. Ψ_{pd} and Ψ_l were measured using a pressure chamber.

Terminal shoots froze in April, 2006 forcing a second flush from axillary buds that produced a pattern of four opposite co-dominant shoots. On May 16, 2006 after growth had progressed, leaf number, leaf length and rachis length were collected on stems throughout the canopy. Additionally, trunk diameters, at 30 cm above ground surface, were measured for all trees in the study at the end of the growing season.

Leaf relative water content (RWC), of selected trees in each block, was measured on August 6, 17 and 24, 2006 as described by Dichio et al. (2005) with the exception that leaves were hydrated by immersion rather than allowing only petiole uptake of water. The equation

$$RWC = \frac{FM - DM}{SM - DM}$$

was used to calculate *RWC* where: *FM* = fresh mass, *DM* = dry mass and *SM* = saturated mass. Ψ_{pd} was measured prior to collection of leaves for RWC. Upon completion of the Ψ_{pd} measurements, a single leaf from each tree was collected very rapidly and placed in sealed plastic bags. These leaves were then immediately transported to the laboratory at ARDEC (approximately 0.5 km away) and weighed to determine fresh mass. Full hydration and subsequent weighing and drying were conducted in the same laboratory without the constraint of time. RWC measurements on August 6, 2006 included leaves from 13 trees from Blocks 1,2,5,7 and 9, August 17, 2006 included 20 trees from Blocks 1,2,3,4,5,7 and 9 and August 30, 2006 included 30 trees from blocks 1,2,3,4 and 5.

Leaves were collected for mid-day RWC using the same procedure with the exception that Ψ_l was not measured at the time of collection. Instead, RWC was collected to determine if Ψ_{pd} could also be used as a predictor of mid-day drought stress.

Differences in mean leaf growth between treatments were conducted using Student T-tests at the 0.05 significance level. The correlation of RWC to Ψ_{pd} was determined using a general linear regression model (SAS Institute, 2008) at the 0.05 significance level. Stepwise multiple regression analysis was used to model g_s, Ψ_l, VPD, PAR, date and hour of measurement on A_n from Block 1(Ctrl) data. Intrinsic water use efficiency (WUE_i) was determined by regressing g_s on A_n of different treatments and comparing the slopes during drought stress and recovery.

1.3) Results and Discussion

2005

The focus in 2005 was the development of drought stress after a well hydrated spring. Early season irrigation was maintained at the 160/80/40 percent ET_o schedule allowing Block 4 (160 percent ET_o) to maintain a period of greater hydration in comparison to Block 5 (40 percent ET_o). Blocks 1 and 2 were maintained as controls and received the historic 160 percent and 40 percent ET_o irrigation regimes respectively. At all four depths, soil moisture in Block 1 remained above 25 percent with minor fluctuations due to irrigation events (Figure 1.2). Under limited irrigation (40 percent ET_o) and drought, soil moisture in Blocks 2, 4 and 5 all dropped dramatically at the 0.30 m depth and remained below 20% for the duration of the study. Increases in soil moisture in Blocks 4

and 5 were due to recovery irrigation at the completion of the drought cycle. At the three deeper depths, 0.6, 0.9 and 1.2 m, soil moisture showed a gradual but continuous decline for the duration of the growing season.

				Gas Exchange		
Date	Soil Moisture	Predawn Leaf Water Potential	Mid-day Leaf Water Potential	Block	Time	RWC
Apr 18	Site Wide					
May 20	Site Wide					
May 21		All Blocks				
May 28	Site Wide	All Blocks				
May 29				All Blocks	09:24-10:36	
May 31			1,2,4,5	1,2,4,5	08:32-10:07	
Jun 04	All Ash					
Jun 06	All Ash	1,4,5,7,9	1,4	1,4	08:27-15:44	
Jun 12	All Ash	All Blocks				
Jun 18		All Blocks				
Jun 19	All Ash					
Jun 26	All Ash	All Blocks				
Jun 29				1,2,4,5,9	09:49-19:49	
Jul 01		All Blocks		1,2,4,5,9	05:12-07:00	
Jul 11	All Ash	All Blocks				
Jul 15		All Blocks				
Jul 20		All Blocks				
Aug 01		All Blocks				
Aug 04		All Blocks				
Aug 06		All Blocks		All Blocks	08:07-15:15	1,2,5,7,9
Aug 08	All Ash	All Blocks	All Blocks	All Blocks	09:35-17:29	
Aug 09	9	All Blocks				
Aug 10		1,3,4,5,7,9	1,4,5,9	1,4,5,9	10:51-14:16	
Aug 11		5,7,9				
Aug 12		5,7,9	1,5,9	1,5,9	11:52-12:48	
Aug 14		All Blocks		All Blocks	10:37-12:14	
Aug 16		All Blocks		1,3,5,7,9	90:15-12:25	
Aug 17		All Blocks		All Blocks	09:29-14:53	All Blocks
Aug 22		All Blocks		All Blocks	12:36-13:51	
Aug 23	2,5	All Blocks				
Aug 24	2,5	All Blocks		1,2,3,4,5	14:46-16:46	1,2,3,4,5
Aug 26				1,2,3,4,5,9	05:09-16:07	
Aug 28	3,4	All Blocks	1,2,3,4,5,9	1,2,3,4,5,9	13:39-15:31	
Aug 29						1,2,3,4,5
Aug 30	3,4	All Blocks		1,2,3,4,5,7	08:07-10:41	
Sep 02		1,2,3,4,5,7				
Sep 03		1,4		1,2,3,4,5,7	08:34-13:13	
Sep 04		1,2,3,4,5				
Sep 05				1,4	15:05-15:34	
Sep 06		1,4				
Sep 07				1,2,4,5	08:55-09:12	

Table 1.4 2006 data collection schedule. Site wide soil moisture measurements included nuclear density gauge access tubes in all 7 blocks studied. Ψ_{pd} were collected from up to seven trees in each study block. Gas exchange measurements included g_s, A_n, and PAR. Mid-day Ψ_l was collected from individual leaves used for gas exchange measurements when possible.

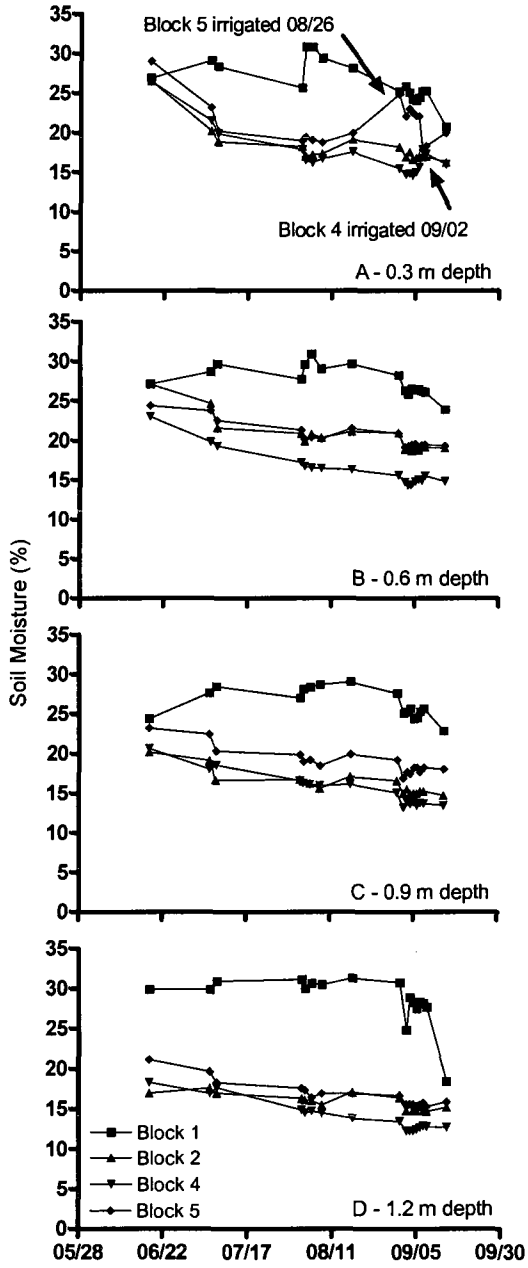


Figure 1.2 Comparison of soil moisture in drought stress treatments (Blocks 2,4,5) to an irrigated control (Block 1) at 0.3(A), 0.6(B), 0.9(C) and 1.2 meters (D) below ground surface in 2005. Soil moisture on any given date is the average of reading from three separate nuclear density gauge access tubes within each block. Tighter clustering of measurements near August 26, 2005 and September 2, 2005 monitored irrigation events used to end the drought stress in Blocks 5 and 4 respectively. Block 2 received 40% and Block 1 received 160% of ET_o throughout the period.

Soil moisture did not increase at depths greater than 0.3 meters in response to the recovery irrigation in Blocks 4 and 5. Decline in Ψ_{pd} corresponded with the gradual drop in soil moisture (Figure 1.3). The August 4, 2005 increase in Ψ_{pd} was due to 6.9 mm of precipitation and cooler temperatures. The undulation of water potentials in Block 2 (40 percent ET_o control) between August 27, 2005 and September 8, 2005 were due to irrigation directly on Block 2 and adjacent Block 1 (160 percent ET_o control). Ψ_{pd} increased in Blocks 4 and 5 were due to recovery irrigation on September 2, 2005 and August 26, 2005 respectively. The 2005 drought induced drop in Ψ_{pd} was greater than drought of similar duration previously recorded by Findley (1999) at ARDEC in 2008 (Table 1.5)

Average treatment block soil moisture, as a compilation of all four depths, and date specific VPD, calculated at dawn (06:00), was regressed on Ψ_{pd}, for all dates when Ψ_{pd} was measured in all treatment blocks (n = 56). Soil moisture had significant control on Ψ_{pd} but accounted for less than 30 percent of the variability ($P<0.0001$, $R^2 = 0.27$). Regressions using depth specific averages of soil moisture, rather than data pooled from all four depths, produced similar results. VPD did not influence Ψ_{pd} ($P= 0.1069$, $R^2 = 0.04$).

	Predawn leaf water potentials (MPa)			
	Aug 6, 1998	Aug 20, 1998	Sep 3, 1998	Sep 17, 1998
40% Irrigation	-0.65	-1.10	-0.85	-1.42
80% Irrigation	-0.65	-0.70	-0.65	-1.38
160% Irrigation	-0.65	-0.70	-0.60	-1.25

Table 1.5 Change in 1998 green ash mean Ψ_{pd} due to three irrigation treatments. Data is from Findley (1999). *n=20 trees per treatment

Trees in control Block 1 responded to normal daily increases in VPD with an afternoon drop in g_s and Ψ_l (Figure 1.4). Average g_s in the well watered control Block 1 reached its peak of 261 mmol $m^{-2}s^{-1}$ just before 10:30. From that point there was a steady decline throughout the day. VPD stayed elevated until afternoon decline in PAR and full recovery of g_s in control Block 1 did not occur until the following morning. Ψ_l in control Block 1 began to decline from a predawn value of –0.37 MPa as soon as PAR and VPD increased and leaf curling was observed beginning approximately 09:30. The declines continued until approximately 16:30, when average Ψ_l reached –1.86 MPa. The decline then reversed and Ψ_l began to increase by 17:00 when PAR and VPD decreased. Ψ_l of bagged leaves, that provided a better estimate of whole tree water status, declined to –1.24 MPa and paralleled the recovery recorded in fully exposed leaves.

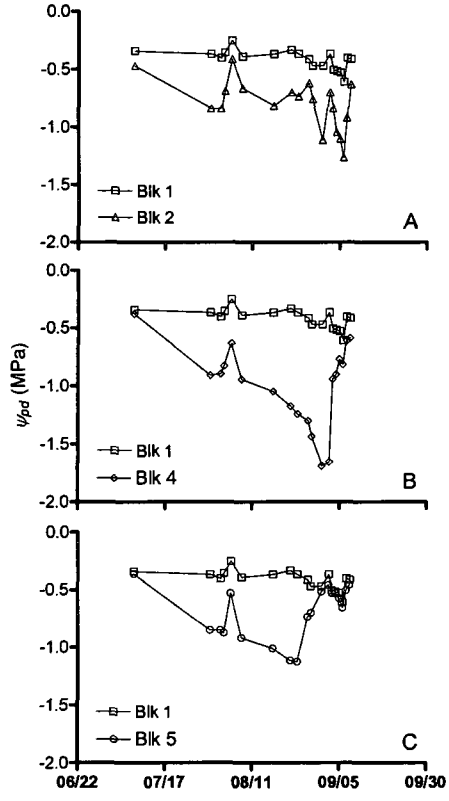


Figure 1.3 Effect of irrigation treatments on Ψ_{pd} of mature green ash trees for 2005. Blocks 1 and 4(B) were irrigated at 160% of ET_o and Blocks 2(A) and 5(C) at 40% of ET_o. Irrigation was withheld from July 17 to September 6, 2005 in Block 4 and July 17 to August 26, 2005 in Block 5. At the end of their respective withholding period blocks 4 and 5 received approximately 50 mm of irrigation.

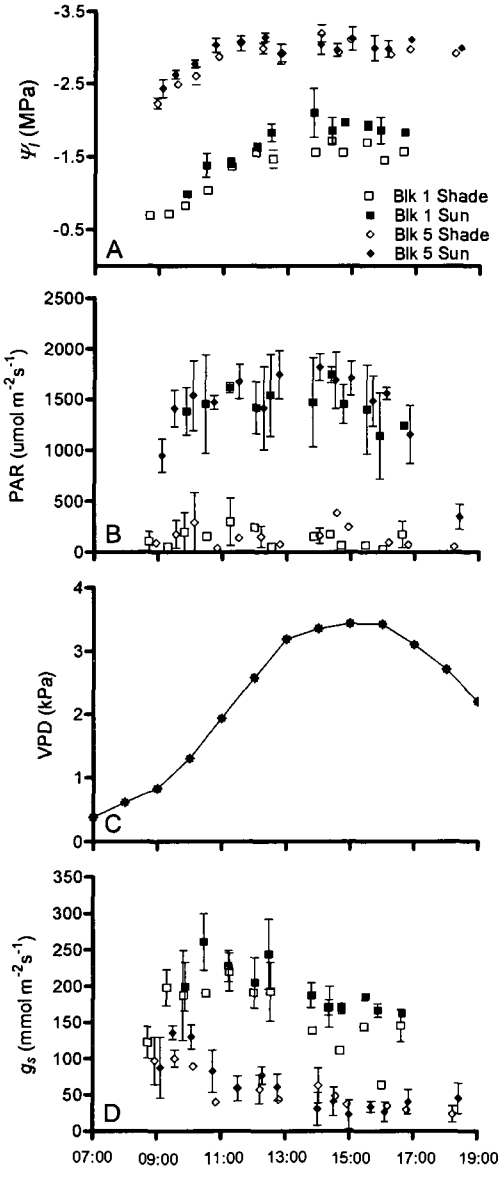


Figure 1.4 Comparison of diurnal trends of g_s, PAR, VPD and Ψ_l in both shaded and fully exposed leaves of drought stressed Block 5 and irrigated control Block 1 green ash trees on August 24, 2005. Points are averages of 2 to 4 individual leaf measurements collected over a 5-10 minute period with error bars representing one standard deviation. Points without error bars are single measurements. These measurements were taken at the peak of drought stress in Block 5, two days prior to recovery irrigation.

Ψ_l and g_s in shade leaves paralleled the effects seen in the sun leaves even with PAR values one or two orders of magnitude lower. Both sun and shade leaves following the same diurnal pattern was a strong indication that VPD was the controlling factor. The response of g_s and Ψ_l in drought stressed Block 5 was similar to control Block 1 but with

greater amplitudes. Average g_s reached its peak of 122 mmol $m^{-2}s^{-1}$ at 09:30 and then declined. The peak was less than 50 percent of the control Block 1 peak and the decline began one hour earlier. By 15:00, g_s declined to 23 mmol $m^{-2}s^{-1}$, 12 percent of the control. Ψ_l began the day with a Ψ_{pd} of –1.13 MPa, declined to –3.03 MPa by 11:00 and remained at that level until after 18:00. Ψ_l of bagged leaves were not significantly different than fully exposed leaves.

On the evening of August 26, 2005, 51 mm of recovery irrigation applied to Block 5, after 41 days of progressive drought stress, produced a rapid response in g_s and Ψ_l (Figure 1.5). On August 27, 2005 Ψ_{pd} for Block 5 was still –0.3 MPa lower than control Block 1. However, Ψ_l measured between 08:00 and 16:30 were statistically equivalent for Block 5 and control Block 1. Stomatal conductance in Block 5 remained depressed in comparison. The diurnal trend of g_s in control Block 1 was not as pronounced on August 27, 2005 as it was on August 24, 2005. Peak average g_s was lower, 236 vs. 261 mmol $m^{-2}s^{-1}$, and it occurred later in the day at 12:00. The lack of a defined diurnal trend in g_s was primarily due to higher morning VPD and lower afternoon values as a result of variable cloud cover. For Block 5, the diurnal trend in g_s, after recovery irrigation, was also less pronounced due to the lack of dramatic mid-day stomatal closure. By August 31, 2005, Ψ_{pd}, mid-day g_s and Ψ_l for Block 5 and control Block1 were equivalent.

Green ash trees in drought stressed Block 4 were subjected to 10 more days of drought stress than Block 5 but responded in a similar manner to Block 5. Block 4 received 50 mm of recovery irrigation on the evening of September 2, 51 days after the initiation of

drought. Prior to recovery irrigation, Ψ_l in Block 4 at mid-day (11:00-14:00) was less than –3.1 MPa and g_s was less than five percent of control Block 1(Figure 1.6). On September 3, 2005 the day after recovery irrigation, Ψ_{pd} in Block 4 was –0.94 MPa compared to a Ψ_{pd} of –0.51 MPa in control Block 1. Ψ_l in Block 4 at 13:00 was statistically equivalent to control Block 1 while average g_s in Block 4 remaining at 46 percent of control Block 1.

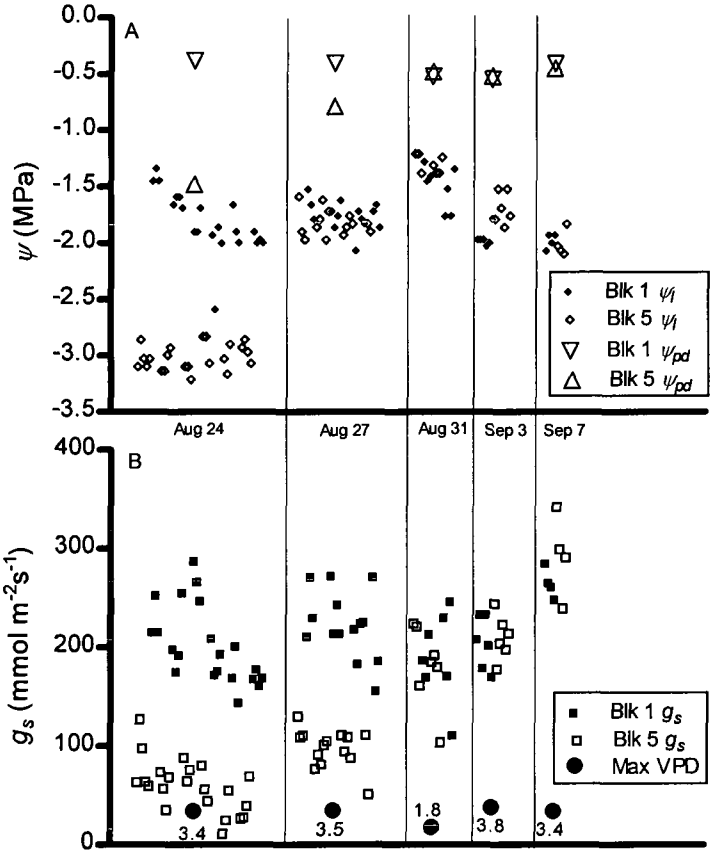


Figure 1.5 Convergence of green ash g_s, Ψ_l and Ψ_{pd} of drought stressed Block 5 with control Block 1 after 25 mm of recovery irrigation was applied to Block 5. August 24, 2005 represents 39 days of drought stress to Block 5. Ψ_l was collected from the same leaves as stomatal conductance measurements. VPD on August 24, 2005, during the peak of drought stress, was similar on September 3 and 7, 2005 and proved that the recovery of g_s and Ψ_l in Block 5 was due to irrigation. Data was collected from one tree in each block.

By September 9, 2005, the pattern had not changed. Ψ_l in Block 4 at 13:30 remained statistically identical to control Block 1, but average g_s had only increased to 50 percent of control Block 1.

Prior to recovery irrigation in Blocks 4 and 5, Ψ_{pd}, g_s and Ψ_l in Block 2 (40 percent of ET_o) were consistently measured at levels between those recorded for control Block 1 and drought stressed Blocks 4 and 5. In addition, recovery irrigation in Block 5 on August 26, 2005 and Block 4 on September 2, 2005 produced a slight increase in Block 2 Ψ_{pd} (Figure 1.6). The response in Block 2 to irrigation in other blocks showed that tree roots extended beyond the block boundaries. After recovery irrigation was completed in the drought stressed Blocks 4 and 5, Block 2 continued to receive irrigation at 40 percent ET_o, and by September 9, 2006 exhibited the greatest level of drought stress. Consequently, Ψ_{pd} in Block 2 remained 0.33 to 0.65 MPa lower than control Block 1, g_s remained 58 to 74 percent of control Block 1, and Ψ_l remained 0.45 and 0.99 MPa below control Block 1.

The 48 day drought stress in 2005 provided a better understanding of site conditions at ARDEC that supported the more extensive research in 2006.

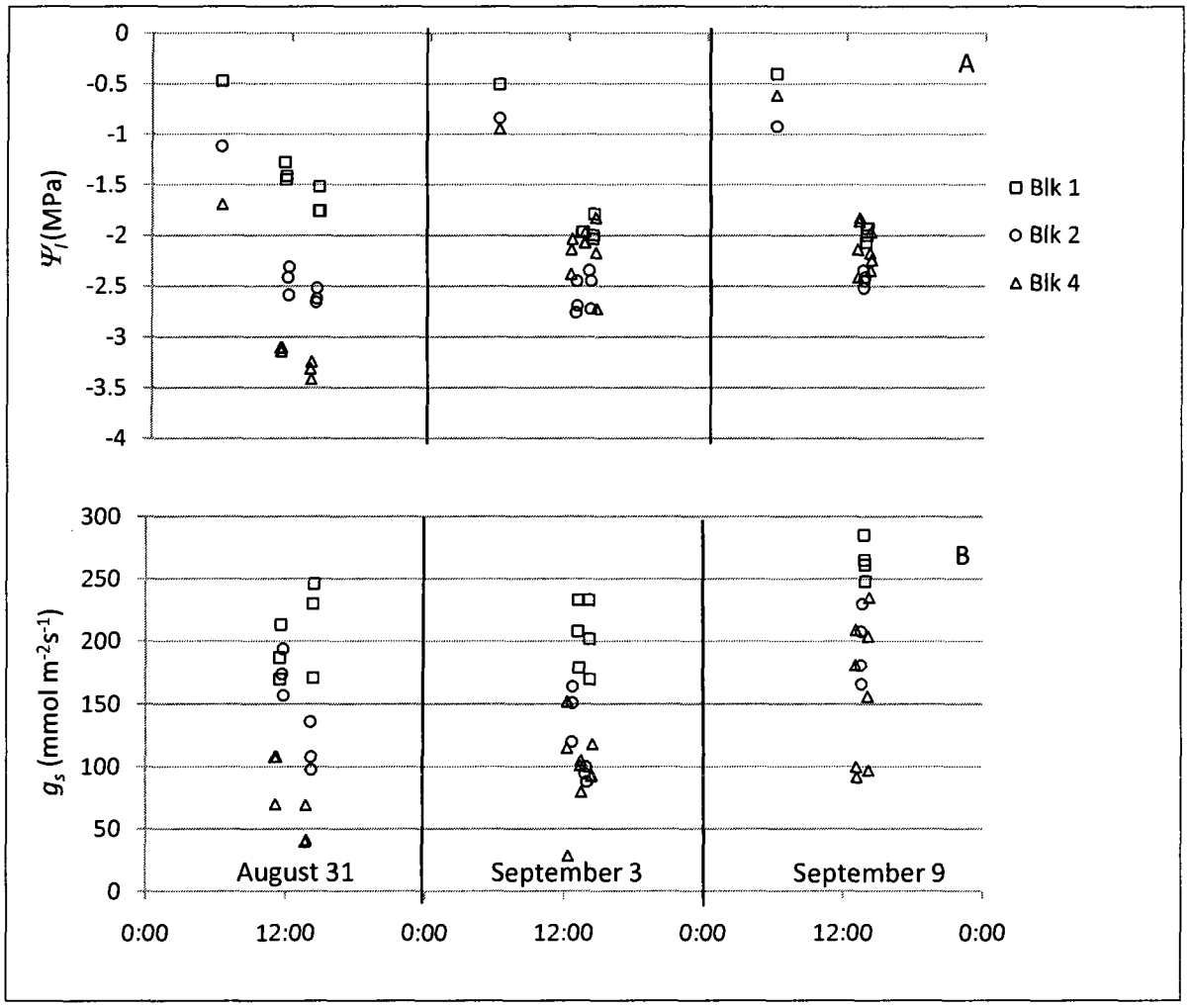


Figure 1.6 Convergence of green ash g_s and Ψ_l for drought stressed Block 4, and control Blocks 1 and 2 between August 31, 2005 and September 9, 2005. Ψ_l at 06:00 (A) represents Ψ_{pd} and shows the rebound in water potential of tree 2E on September 3, 2005 immediately after irrigation of Block 4 on September 2, 2005. The increase in Ψ_{pd} is followed by a gradual decline until September 6, 2005. Data was collected from one tree in each block.

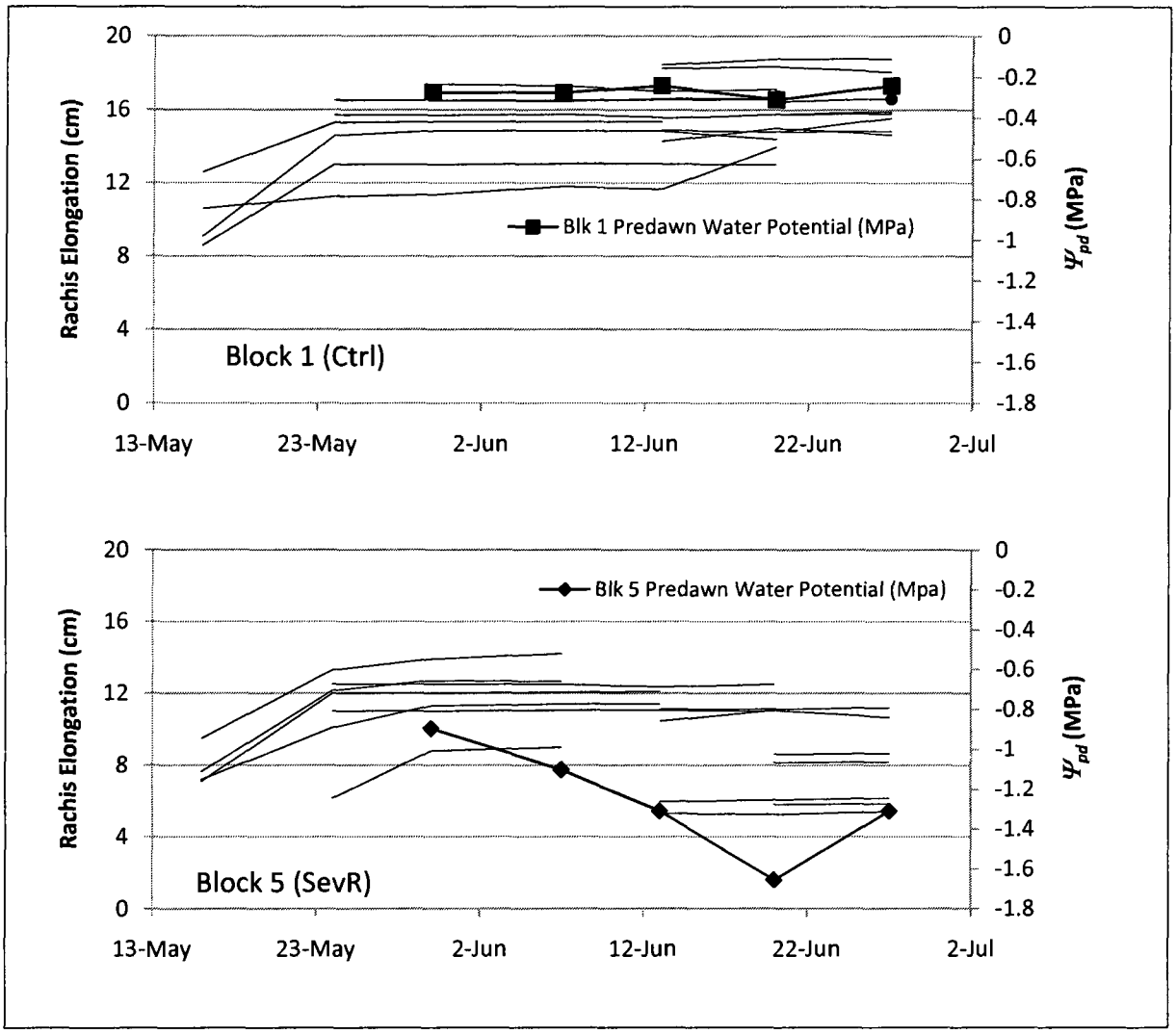


Figure 1.7 Cessation of 2006 leaf expansion in Block 5 (SevR) as compared to Block 1 (Ctrl). Ψ_{pd}, presented on a secondary axis, show that drought reduced overall growth in Block 5 (SevR) when compared to the Block 1 (Ctrl) but did not cause the cessation of growth. Each line represents growth measurements from a single leaf.

2006 Spring Leaf Growth

The winter of 2005-2006 was the driest on record, providing the conditions for early season drought stress in the green ash trees at ARDEC and by May 30, 2006 leaf expansion stopped in all blocks measured (Figure 1.7). Data presented for Block 5 (SevR) are indicative of the lesser growth in all drought stressed treatment blocks relative to the control. Growth of the largest leaves in the upper canopy of the drought stressed

trees in Blocks 2 (SevR), 4 (SevR) and 5 (SevR) were statistically equivalent with an average length of 11.7 cm. Average leaf size in the drought stressed trees was 72 percent of control Block 1 (P<0.000003). In addition, leaves in the upper canopy of Block 1 (Ctrl) consistently had seven or eight pairs of leaflets while leaves in drought stress blocks (SevR) had as few as three pairs of leaflets. In drought stressed trees, after the initial flush of growth many of the terminal leaflets did not fully mature. The combination of smaller and fewer leaves produced a much smaller and more open canopy.

Neither Abrams nor Shumway described the determinant growth pattern that is exhibited in the mature green ash trees at ARDEC. Also, their research with green ash seedlings showed a response to drought stress at Ψ_{pd} of –0.6 MPa. As the reader will see in later sections, those values are only slightly less negative than the values measured in well watered control trees (Block 1) at ARDEC on May 21, 2006 and suggest that even slight decreases in Ψ_{pd} are sufficient to reduce shoot elongation and leaf expansion.

2006 Water Potentials and Soil Moisture

Relative water content (RWC) correlated well with Ψ_{pd} but coefficient of variation (R^2) varied with collection dates (Figure 1.8). Slopes of each regression were also similar showing that the RWC-Ψ_{pd} relationship was consistent throughout the measurement period. Mid-day RWC measurements collected on August 17, 2006 had a split relationship (Figure 1.9). Trees with Ψ_{pd} above -2.35 MPa had a poor correlation with mid-day leaf RWC (R^2 = 0.26) while trees with Ψ_{pd} below -2.35 MPa correlated well

with mid-day RWC ($R^2 = 0.97$). As RWC dropped below 70 percent and Ψ_{pd} dropped below -2.35 MPa, depressed transpiration dramatically reduced the amount of water lost during the photosynthetically active period of the day.

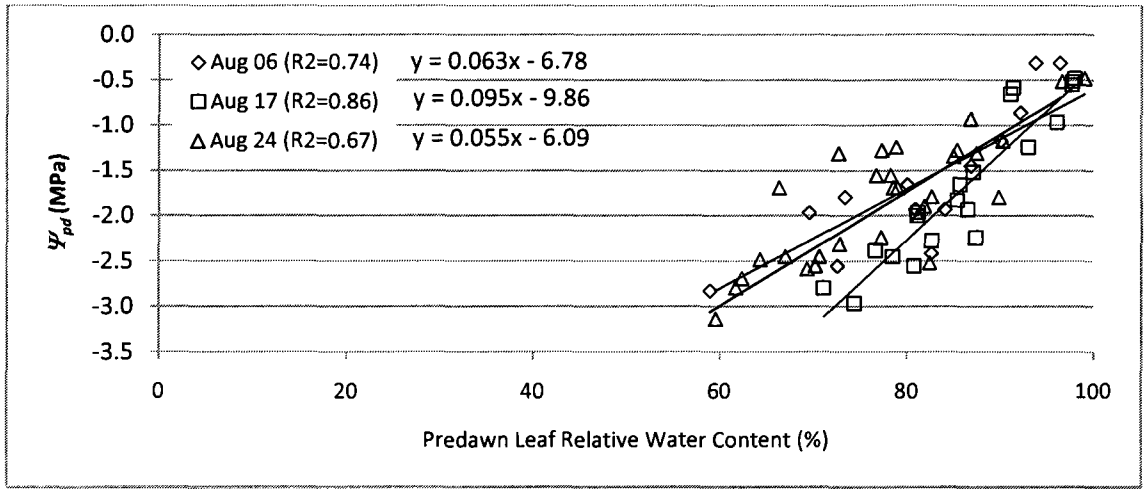


Figure 1.8 Correlation of predawn leaf RWC vs. Ψ_{pd} for green ash tress receiving either drought stress or irrigation. August 6, 2006 represented 13 trees from blocks 1, 2, 5, 7 and 9. August 17, 2006 represented 20 trees from blocks 1, 2, 3, 4, 5, 7 and 9. August 24, 2006 represented 30 trees from blocks 1, 2, 3, 4 and 5.

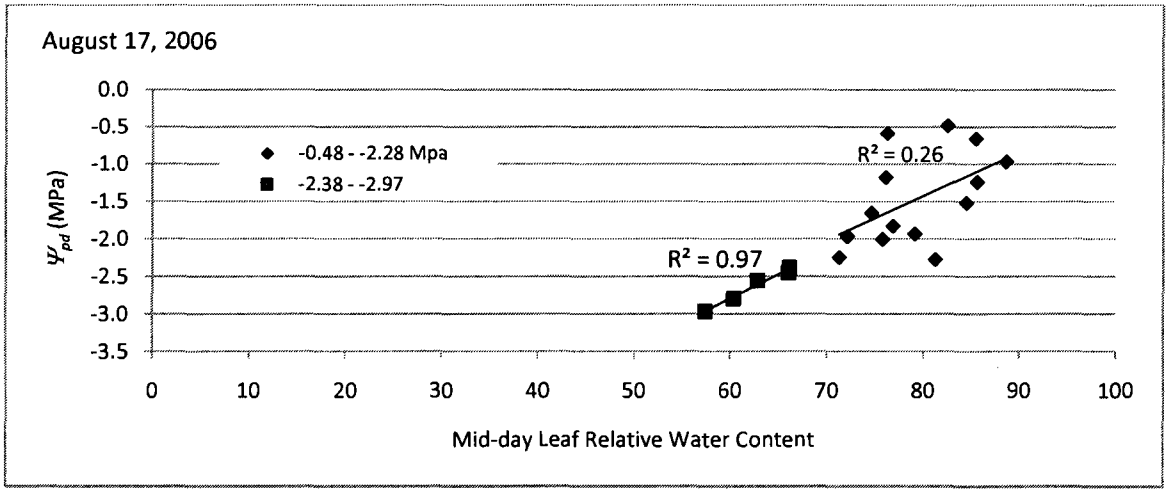


Figure 1.9 Correlation of mid-day leaf RWC vs. Ψ_{pd} for 19 green ash tress receiving either drought stress (Blocks 2,3,4,5,7 and 9) or irrigation (Block 1). Separating the data to account for Ψ_{pd} above or below -2.35 MPa showed the higher R^2 with mid-day RWC and trees under greater drought stress.

Block 1(Ctrl) was irrigated regularly from May 14, 2006 through September 4, 2006 and provided a baseline for well hydrated trees (Figure 1.10). Minor fluctuations in Ψ_{pd} were due to the exact timing of irrigation that was dependent on calculated bluegrass ET_o and the availability of irrigation water. Throughout the growing season soil moisture was maintained near 25% at all depths. The highest soil moisture recorded was 28.5% and represented saturated soils. Through July, 2006, Ψ_{pd} was consistently greater than -0.32 MPa. During late August and early September, Ψ_{pd} dipped to -0.72 MPa but those fluctuations were isolated and the mean Ψ_{pd} was maintained at -0.44 MPa.

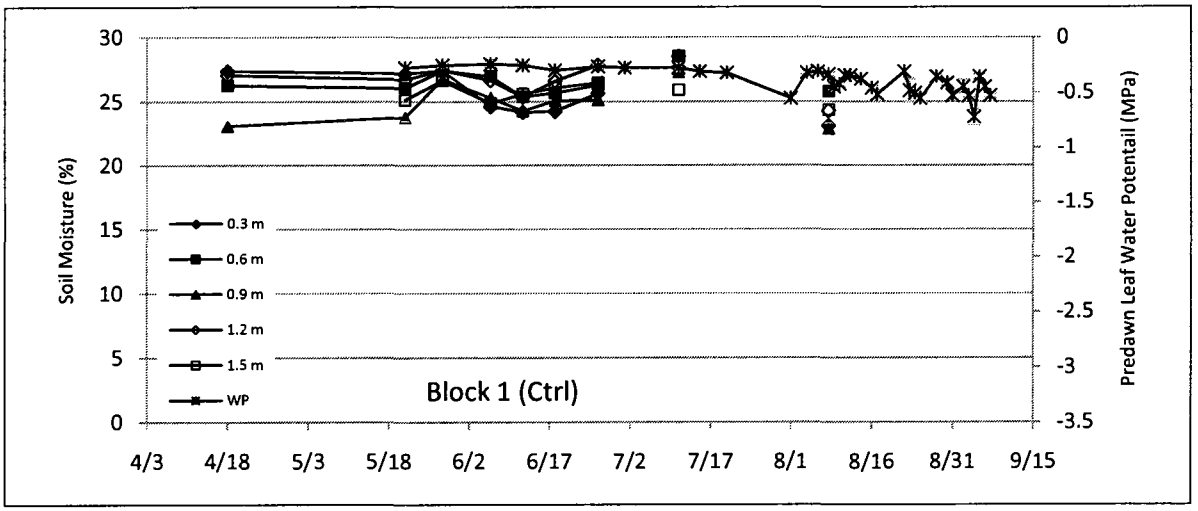


Figure 1.10 Effects of consistent irrigation on soil moisture and green ash Ψ_{pd} throughout the growing season. Soil moisture to a depth of 1.5 meters was maintained above 24 percent and represents near saturation conditions. Ψ_{pd} between -0.32 and -0.72 MPa represents non-stressed conditions.

Block 9 (ModR) soil moisture, to a depth of 1.5 meters, was depleted at the beginning of the season and never exceeded 19.1 percent (Figure 1.11). By early June, 2006 bluegrass turf in Block 9 was dormant, signaling the lack of available water down to a depth of approximately 0.3 m. The 14 percent soil moisture measured at that depth

reflects the wilting point for grass in the clayey soils at ARDEC. Soil moisture in deeper soils down to 1.5 m also dropped to approximately 14 percent.

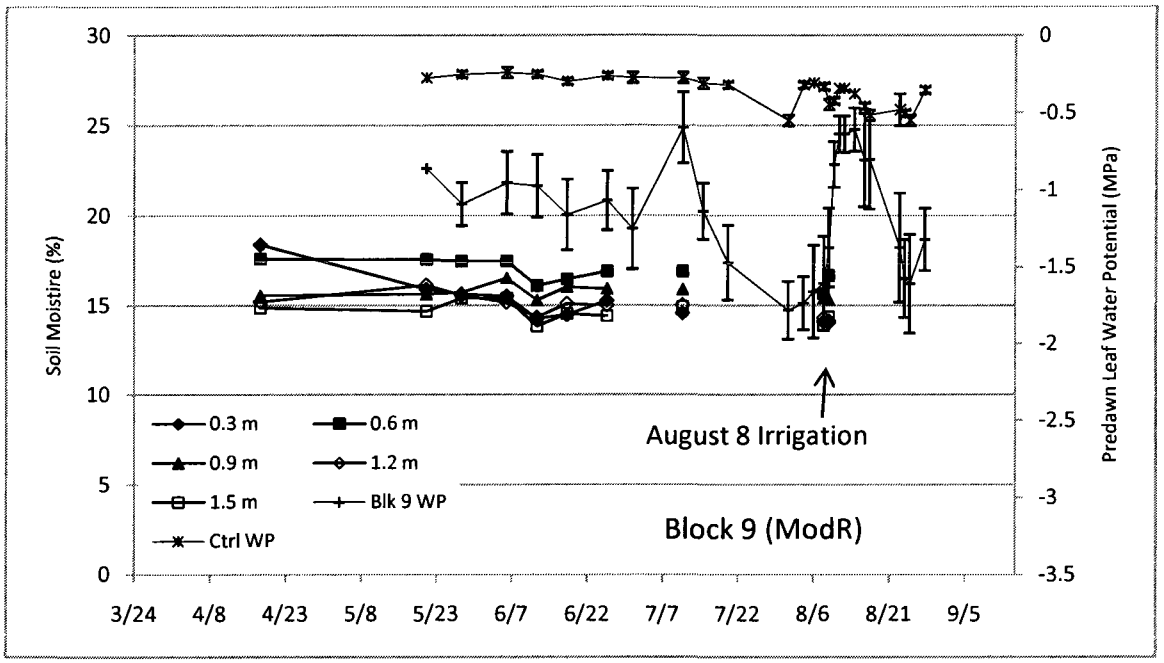


Figure 1.11 Effect of drought and recovery irrigation on soil moisture and Ψ_{pd} in green ash (Block 9) subjected to moderate drought stress (ModR). That rapid increase in Ψ_{pd} (Blk 9 WP) to 26 mm of irrigation on August 8, 2006 raised average Ψ_{pd} in the block to near control levels (Ctrl WP). The heterogeneity of the irrigation application affected soil moisture measurements at 0.3 m and no increase was recorded. Error bars are one standard deviation.

These consistently low soil moistures near the wilting point indicate that tree roots were using soils deeper than 1.5 m to maintain ET. Ψ_{pd} dropped gradually in Block 9 through June, 2006, but by late July, 2006 began to decline more rapidly and on August 1, 2006 a single tree within the block reached a Ψ_{pd} of -2.03 MPa . By August 6, 2006 Block 9 was considered moderately stressed when Ψ_{pd} in all seven interior trees ranged between -1.10 and -1.97 MPa (mean = -1.66 MPa). On the evening of August 8, 2006 approximately 26mm of recovery irrigation was applied to Block 9. Response to the recovery irrigation was measureable the following morning (Figure 1.1) and Ψ_{pd} increased until, on August 11, 2006, the average for the block reached –0.61 MPa. At

to drop as the lack of continued irrigation allowed soil moisture to decline. Twenty-six millimeters of water was sufficient to increase moisture in the top 25 cm of soil by 10 percent, however soil moisture, even at the 0.3 m depth, did not show an increase from the irrigation. The response in Ψ_{pd} confirmed the effects of recovery irrigation but the limited spatial control of only three nuclear density gauge access tubes was insufficient to assess that heterogeneity of the irrigation.

Blocks 2, 3, 4 and 5 (SevR) responded similarly to drought over the course of the growing season. (Figures 1.12 and 1.13) and followed the general pattern experienced in Block 9 (ModR). At the beginning of the 2006 growing season soil moisture at 0.3 m was greater than 1.2 m with some variability between blocks. By the middle of June, 2006 soil moisture at both depths and in all blocks coalesced at approximately 15 percent. Soil moisture stayed consistently low in all SevR treatment blocks until recovery irrigation. Ψ_{pd} dropped gradually for all SevR treatment blocks and by early August, 2006 reached a point described as 'severe.' From early August, 2006 until recovery irrigation was applied, minor fluctuations in Ψ_{pd} were characteristic of the severe drought conditions.

Recovery irrigation of 51 mm applied on August 23, 2006 to Blocks 2 and 5 resulted in an increase of Ψ_{pd} and soil moisture (at 0.3 m) the following morning. By August 28, 2006, Ψ_{pd} in both Blocks 2 and 5 was statistically equivalent to the Block 1 control. Blocks 3 and 4 also responded to irrigation of Blocks 2 and 5 with a slight increase in Ψ_{pd} and signaled the end of severe drought stress. On August 29, 2006, 51mm of irrigation

was applied to Blocks 3 and 4 resulting in the same increase of Ψ_{pd} and soil moisture (at 0.3 m) the following morning. However, the soil moisture increase in Blocks 3 and 4 was only a few percent. This muted response was similar to Block 9 (ModR). Regardless of the soil moisture measurements, the Ψ_{pd} increase in Blocks 3 and 4, to control levels, show that complete recovery from drought stress was achieved.

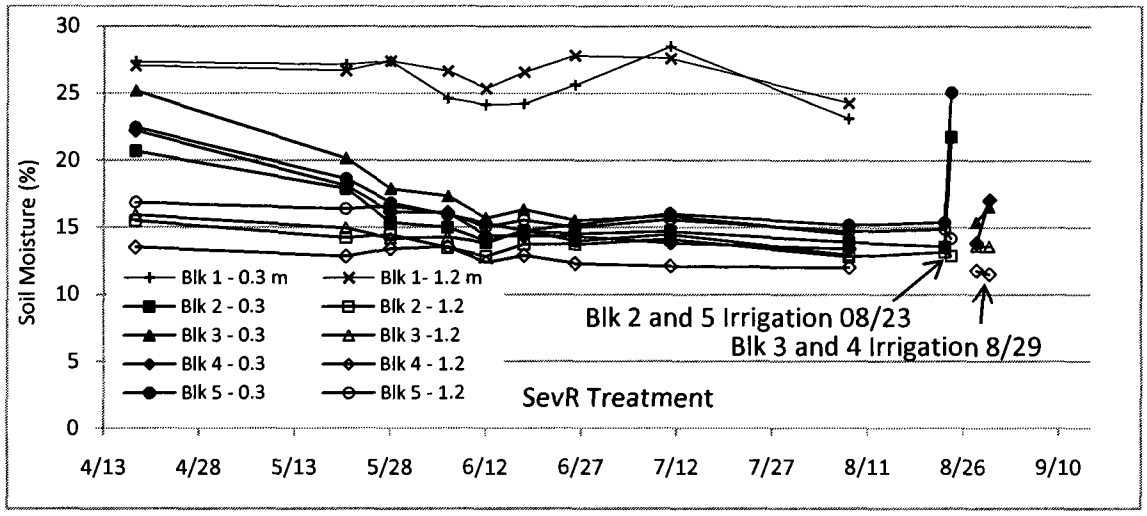


Figure 1.12 Effect of drought and recovery irrigation on soil moisture in green ash treatment Blocks 2, 3, 4 and 5 subjected to severe drought stress (SevR). After 51 mm of irrigation on August 23, 2006 Block 5 and 2 showed an increase in soil moisture as great as 10 percent. The soil moisture response in Blocks 3 and 4 to similar irrigation on August 29, 2006 was not as large.

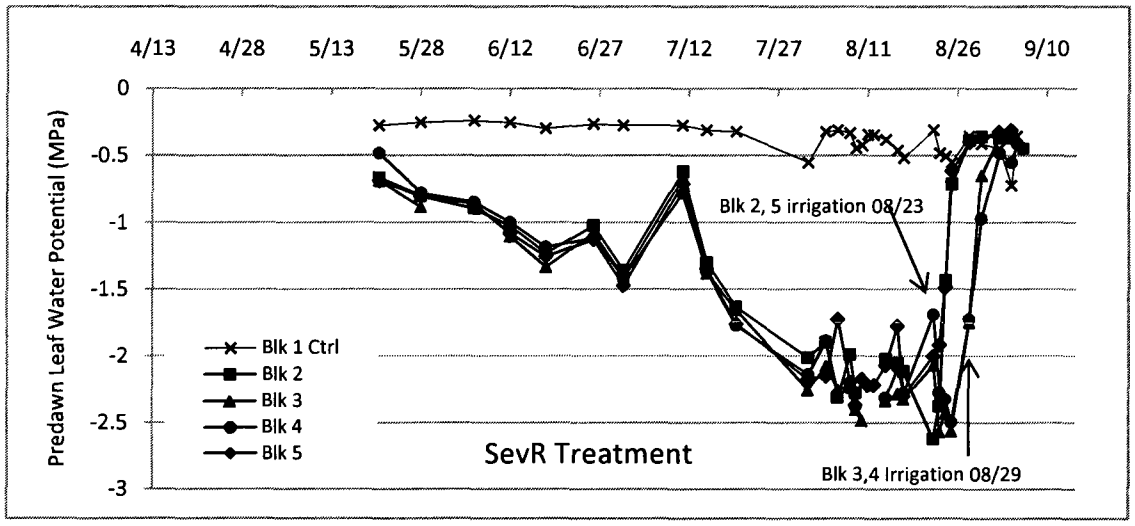


Figure 1.13 Effect of drought and recovery irrigation on Ψ_{pd} in green ash treatment Blocks 2, 3, 4 and 5 subjected to severe drought stress (SevR). That increase in Ψ_{pd} in all treatment blocks to 51 mm of irrigation on either August 23 or 29, 2006 raised average Ψ_{pd} to near control levels (Ctrl).

Treatment Block 7 (ModNR), responded to drought with an early season drop in soil moisture but Ψ_{pd} did not decline in the profile, as it did in other treatment blocks (Figure 1.14). Soil moisture declined to approximately 14 percent at all five depths in Block 7 by August 8, 2006. Ψ_{pd} gradually declined through the growing season but the drop did not reflect the low soil moisture. By August 23, 2006 the average Ψ_{pd} in Block 7 reached a low of –1.36 MPa but did not decline further. On August 30, 2006 a minor increase in Ψ_{pd} of 0.3 MPa was associated with recovery irrigation in neighboring Block 4 and Ψ_{pd} continued to rise into September, 2006.

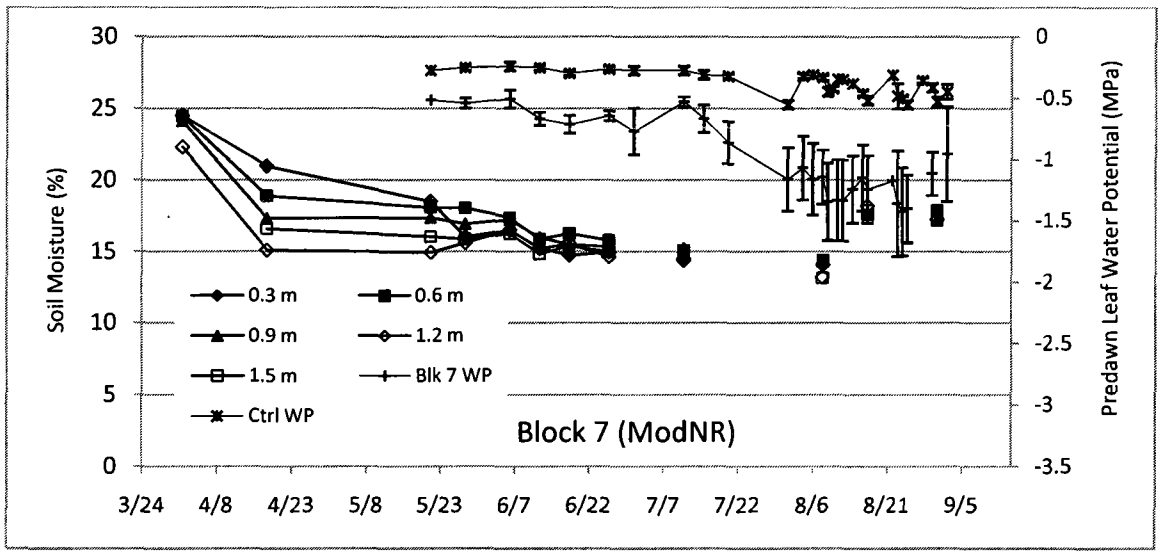


Figure 1.14 Effect of drought on soil moisture and Ψ_{pd} in green ash treatment Block 7, subjected to moderate drought stress without recovery irrigation (ModNR). Early season declines did not continue through August, 2006 as seen in other treatment blocks. That minor increase in Ψ_{pd} (Blk 7 WP) was due to irrigation in adjacent Block 4 on August 29, 2006. Error bars are one standard deviation.

2006 Leaf Gas Exchange

Block 1 (Ctrl) remained non-stressed throughout the growing season and provided the baseline to evaluate leaf gas exchange in all other treatment blocks subjected to drought

stress. On June 29, 2006 when VPD reached 4.0 kPa there was a distinct mid-day depression in g_s and A_n that began between approximately 10:00 and 11:30 and continued past 15:30 (Figure 1.15). On August 6, 2006, a day with max VPD of only 2.7 kPa, mid-day depression in A_n and g_s did not occur. The lack of mid-day depression in Block 1 (Ctrl) became the normal condition through September 7, 2006. A comparison of morning (08:30 - 11:00) and mid-day (12:00 to 14:45) measurements from August 10, 2006 through September 7, 2006 showed no statistical difference between time of day for either g_s or A_n, even though VPD was statistically different between the two time periods (P<0.01). Also, there was no correlation that would suggest control of g_s by VPD (R^2 = 0.14, P=0.147). Variability in measurements of g_s between leaves, for any specific period of time, was generally equal to or greater than means of those measurements on different days. Although the controls on g_s are well described in the literature (Niinemets et al., 2004; Wullschleger et al., 2000; Thomas and Eamus, 1999; Aphalo and Jarvis, 1993a-b; Jones, 1998), the low R^2 value suggests that that variability between individual leaf measurements is mostly unexplained. Heterogeneity in canopy dynamics and biological control of stomata, not associated with drought stress, had a greater control on g_s than incident PAR and ambient VPD.

Stepwise multiple regression analysis was used to model g_s, Ψ_l, VPD, PAR, date and hour of measurement on A_n from Block 1(Ctrl). The entire data set of gas exchange measurements with PAR > 500 and g_s values less than 262 mmol $m^{-2}s^{-1}$ was used. PAR and g_s together account for most of the variability in A_n (R^2 = 0.79, P<0.0001, A_n = $0.0379g_s + 0.0034\text{PAR} - 1.38$).

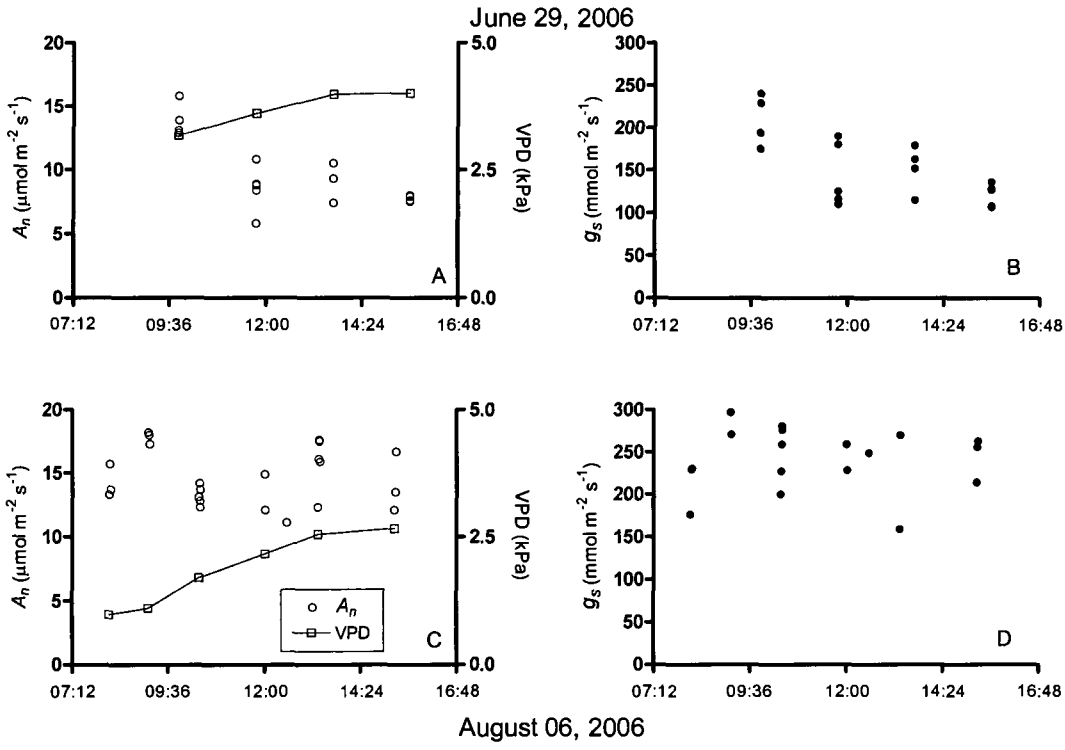


Figure 1.15 Diurnal effect on A_n and g_s in well watered green ash (Blk 1 Ctrl) on Jun 29, 2006 and August 06, 2006. VPD increased until late afternoon as is typical of a clear Colorado day. Mid-day depression of both A_n (A) and g_s (B) occurred on June 29, 2006. Lower VPD on August 6, 2006 did not induce mid-day depression of A_n (C) or g_s (D). Each point represents a single measurement.

The use of the PAR threshold and the g_s threshold removed the factors of shaded leaves and measurements where g_s was beyond the photosynthetic maximum (Hogg et al., 2000). Adding VPD and date of measurement to the model produced a marginal improvement ($R^2 = 0.83$) but the improved correlation was not sufficient to justify their inclusion. Although the controlling factors for g_s were not well explained by the data, g_s did have a direct and measurable effect on A_n. The relationship between g_s and A_n includes a complex interaction of environment (wind, temp, radiation, humidity), soil water status and plant water status which in turn has influence on CO_2 feedback through leaf internal CO_2 concentrations or assimilation (Jones 1998). Within that dynamic, g_s and PAR explained fully 79% of the variation of A_n.

The relationship between g_s and A_n provided an estimate of intrinsic water use efficiency (WUE_i), defined as minimum g_s for maximum A_n (Arndt et al., 2001; Bota et al., 2001). On August 16, 2006, at approximately 10:45 (VPD ≈ 1.50), A_n in Block 1(Ctrl) ranged between 15.8 and 17.8 umol $m^{-2}s^{-1}$ (PAR = 1744 - 2272 umol $m^{-2}s^{-1}$) with g_s between 222 and 264 mmol $m^{-2}s^{-1}$, representing the peak in A_n. By excluding low value of PAR that represented shade leaves, and values of g_s higher than those corresponding to maximum A_n, the relationship between A_n on g_s, in Block 1(Ctrl), could be accessed through linear regression (Figure 1.16). The data show that g_s of approximately 262 mmol $m^{-2}s^{-1}$ provided optimal WUE_i.

Treatment block specific A_n and g_s regressions (PAR > 500 μmol $m^{-2}s^{-1}$) show similar R^2 values for all blocks receiving drought stress (Table 1.6). Prior to the initiation of recovery irrigation all treatment blocks had similar slopes that were significantly steeper than Block 1(Ctrl).

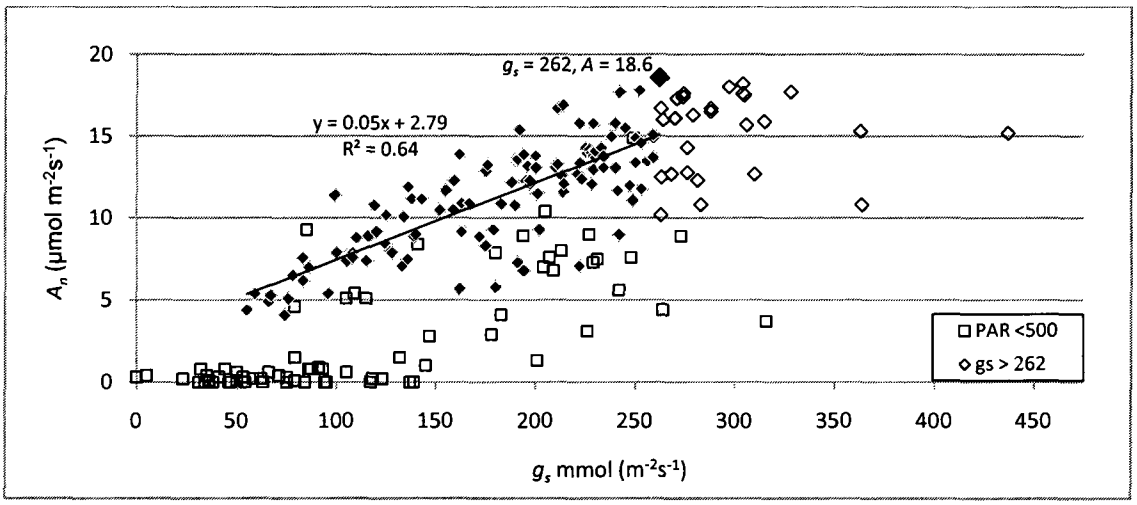


Figure 1.16 WUE_i in green ash as defined by full season measurements of g_s at maximum A_n for control Block 1. The trend line and associated R^2 represents a linear regression for all values of A_n with PAR >500 μmol $m^{-2}s^{-1}$ and g_s < 263 mmol $m^{-2}s^{-1}$. PAR values of 500 μmol $m^{-2}s^{-1}$and lower represented shaded leaves.

The increased slope is a measure of increased WUE_i where A_n is maintained even though g_s experienced a relatively greater reduction for water conservation. After recovery irrigation, the slopes were reduced in all treatment blocks, except Block 3 (SevR), due to a rapid recovery in g_s levels without a complete recovery in A_n to early season values.

Block	R^2	Equation		
1 (Ctrl)	0.64	$A_n = 0.05g_s + 2.79$		
			R^2	Equation
	During Stress Period		After Recovery Irrigation	
2 (SevR)	0.93	$A_n = 0.09g_s + 0.21$	0.58	$A_n = 0.07g_s + 1.08$
3 (SevR)	0.86	$A_n = 0.08g_s + 0.41$	0.77	$A_n = 0.09g_s - 0.07$
4 (SevR)	0.90	$A_n = 0.09g_s + 0.20$	0.44	$A_n = 0.04g_s + 1.95$
5 (SevR)	0.87	$A_n = 0.09g_s + 0.05$	0.36	$A_n = 0.04g_s + 3.29$
7 (ModNR)	0.82	$A_n = 0.08g_s + 0.12$		
9 (ModR)	0.93	$A_n = 0.09g_s + 0.35$	0.90	$A_n = 0.07g_s + 0.66$

Table 1.6 Changes in green ash WUE_i for all treatment blocks subjected to drought stress. The steeper slopes of drought stressed blocks show relatively higher A_n as stomatal control was induced to conserve water. After recovery irrigation, shallower slopes show that A_n did not recover from stress as quickly as g_s. All stressed treatments had greater WUE_i than the control Block 1. Data include all measurements with PAR > 500 μmol $m^{-2}s^{-1}$and g_s less than 262 mmol $m^{-2}s^{-1}$.

Block 9 (ModR)

The early application of recovery irrigation on Block 9 (ModR) allowed for a recovery response in A_n and g_s followed by a second cycle of drought stress. In Block 9 (ModR) on May 9, 2006, maximum values for A_n and g_s were 7.4 umol $m^{-2}s^{-1}$ and 55 mmol $^{-2}s^{-1}$ respectively in trees with Ψ_{pd} (–0.93 to –1.17 MPa) sufficient to reduce growth. Average g_s and A_n were respectively 38 percent and 68 percent of control Block 1 (Figure 1.17). The reduced g_s produced a much steeper regression slope and higher WUE_i than Block 1 (Ctrl). On August 6 and 8, 2006 maximum A_n and g_s in Block 9 (ModR) were essentially the same as May 29, 2006, 6.0 umol $m^{-2}s^{-1}$ and 64 mmol $m^{-2}s^{-1}$ respectively. However, mid-day depression of g_s reduced A_n as low as 0.2 μmol m^{-2} s^{-1}(μ=2.5, SD=1.8,

n=25) On August 16, 2006 eight days after the August 8, 2006 recovery irrigation, maximum values for A_n and g_s, 15.1 umol $m^{-2}s^{-1}$ and 229 mmol $m^{-2}s^{-1}$ respectively, were approximately 85% of Block 1 (Ctrl) but mid-day A_n and g_s were statistically equivalent to the period of peak stress, August 6 and 8, 2006. The recovery of A_n and g_s to levels greater than those measured in the spring suggests that early season growth inhibition may not permanently reduce A_n and g_s capacity. However, after a period of drought stress A_n and g_s were still highly sensitive to mid-day depression.

Blocks 2 and 5 (SevR)

Block 2 and Block 5, severe drought stress with recovery treatments, achieved lower Ψ_{pd} and maintained stress for an additional two weeks past Block 9 (ModR). The results for Blocks 2 and 5 were statistically equivalent and were combined for treatment analysis. On May 29 and 31 (08:32-10:01) maximum values in Blocks 2 and 5 (SevR) for A_n and g_s, 10.6 umol $m^{-2}s^{-1}$ and 141 mmol $m^{-2}s^{-1}$ respectively, were higher than those recorded for Block 9 (ModR) and consistent with the higher average Ψ_{pd} in Block 2 and 5 (SevR) of –0.78 MPa. Although maximum A_n and g_s for Blocks 2 and 5 (SevR) were lower than Block 1 (Ctrl), the means were statistically equivalent (Figure 1.18).

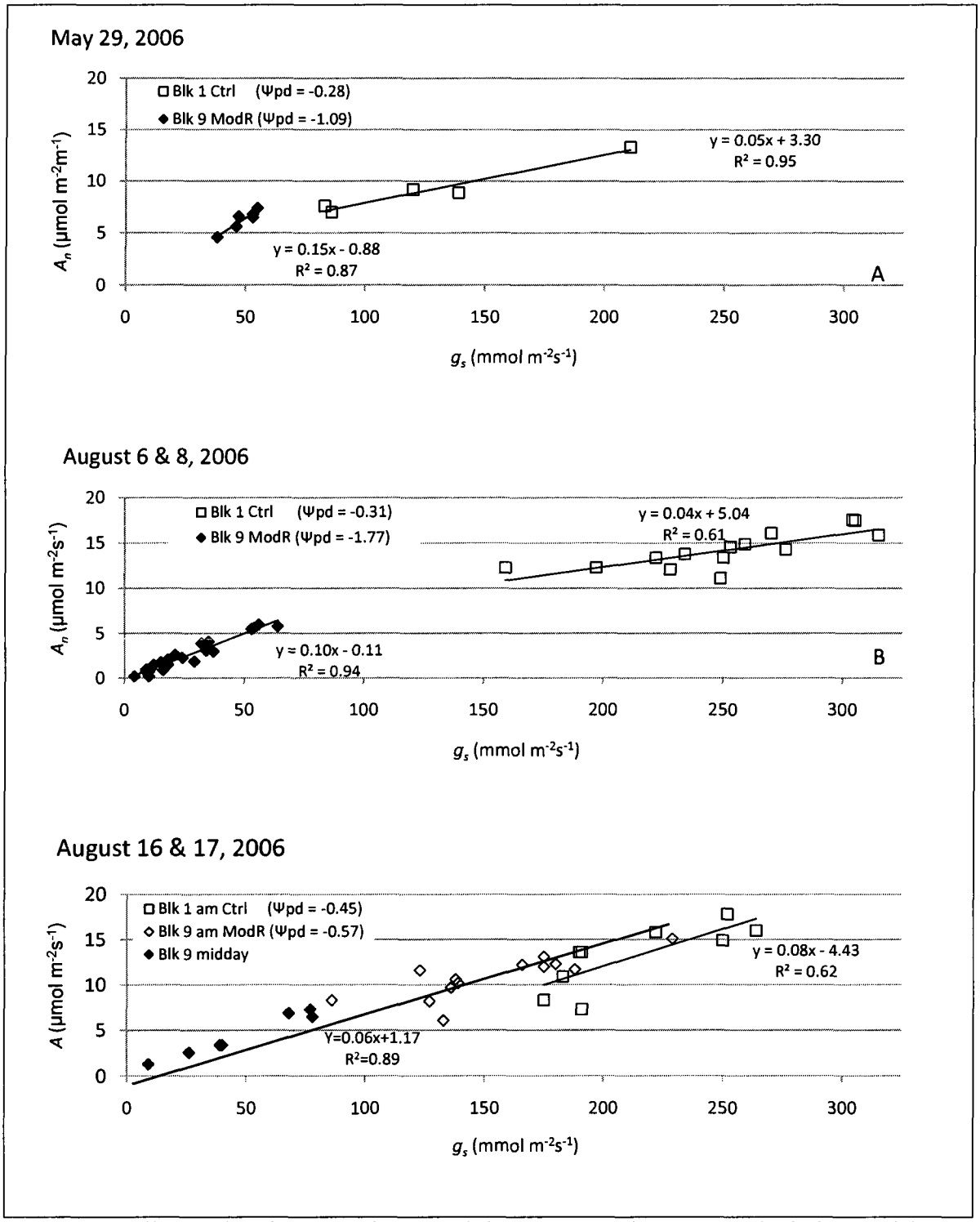


Figure 1.17 Effects of drought stress and recovery irrigation on A_n and g_s in green ash Block 9 (ModR) compared to Block 1 (Ctrl). Early season (A) conditions reduced A_n and g_s but not as severely as peak drought stress on August 6 and 8, 2006 (B). Morning and mid-day A_n and g_s increased dramatically (C) after application of recovery irrigation on August 8 but were still lower than Block 1 (Ctrl). Mid-day depression of A_n and g_s still occurred (C) but not at the levels measured at peak drought stress (B). Regression slopes show WUE_i increased at peak moderate drought stress but returned to control levels after irrigation.

On August 17, 2006, prior to recovery irrigation, the reduced Ψ_{pd} in Blocks 2 and 5 (SevR) caused a dramatic decrease in morning A_n and g_s with an even greater drop at mid-day to levels approaching zero. A_n and g_s in Blocks 2 and 5 (SevR), near peak severe drought stress, were lower than those measured near peak moderate stress (August 6 and 8, 2006) in Block 9 (ModR) and reflect the lower Ψ_{pd} reached during the longer drought of the SevR treatment. At the peak of moderate stress Block 9 (ModR) did not exhibit the same afternoon declines in A_n and g_s observed in Blocks 2 and 5 (SevR). On August 28 and 30, 2006, approximately one week after recovery irrigation, average morning A_n and g_s recovered to 7.0 umol $m^{-2}s^{-1}$ and 95 mmol $m^{-2}s^{-1}$ respectively and were statistically equivalent to May 29 and 31, 2006. Average mid-day measurements of both A_n and g_s, however, were both depressed by 55 percent compared to May 29 and 31, 2006. The inability of A_n and g_s to rebound above early season levels is in contrast to the response in Block 9 (ModR). Early season A_n and g_s were similar in Blocks 2, 5 and 9 and the inability of Blocks 2 and 5 to exceed those values after recovery irrigation suggests that the longer drought stress permanently affected A_n and g_s. Unlike Block 9 (ModR), early season Block 2 and 5 (SevR) WUE_i was similar to Block 1 (Ctrl). With the onset of severe stress in Blocks 2 and 5, dramatic afternoon declines in A_n and g_s altered the stated concept of WUE_i. Stomatal conductance reached the physical minimum and any A_n was a result of gas exchange that could not be controlled. After recovery irrigation, Block 2 and 5 g_s returned to the range of effective control and WUE_i increased over Block 1 (Ctrl).

Blocks 3 and 4 (SevR)

Blocks 3 and 4 (SevR) were subjected to severe drought stress for six days longer than Blocks 2 and 5 (SevR), but response in Ψ_{pd}, A_n and g_s were equivalent. Despite the reduced leaf growth described previously (Figure 1.7) diurnal trends in A_n and g_s on June 29, 2006 for Block 4 and Block 1 (Ctrl) were similar and demonstrate that a Ψ_{pd} between -0.28 and -0.78 was within the range indicative of well hydrated trees. Mid-day A_n and g_s in Blocks 3 and 4 (SevR), on August 28 just prior to initiation of irrigation, were the most depressed of all severe drought stress treatment blocks even though Ψ_{pd} were not as low as those measured in Blocks 2 and 5 (Figure 1.19). A_n, between zero and 1.2 umol $m^{-2}s^{-1}$, and g_s, between 0 and 13mmol $m^{-2}s^{-1}$, represented conditions of maximum resistance to water loss. On September 3, four days after irrigation, Ψ_{pd} in Blocks 3 and 4 (SevR) increased to near control levels and average A_n and g_s recovered to approximately 50 percent of early season values. That response was consistent with all Blocks receiving the severe drought stress treatment.

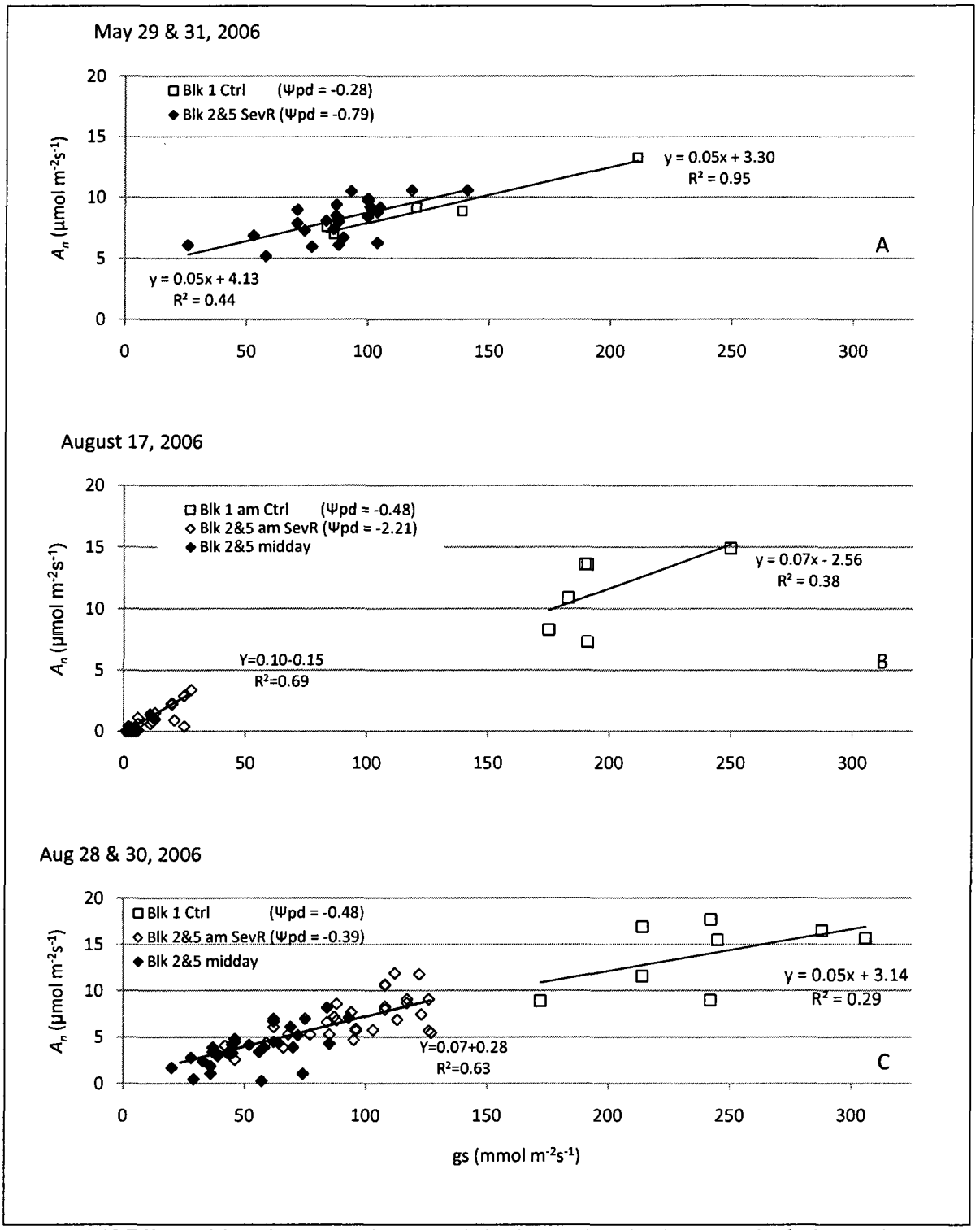


Figure 1.18 Effects of drought stress and recovery irrigation on A_n and g_s in green ash Blocks 2 and 5 (SevR) compared to Block 1 (Ctrl). Early season (A) conditions reduced A_n and g_s but not as severely as peak drought stress on August 17 (B). Morning and mid-day A_n and g_s increased dramatically (C) one week after application of recovery irrigation on August 23, 2006 but were still lower than Block 1 (Ctrl). Mid-day depression of A_n and g_s still occurred (C) but not at the levels measured at peak drought stress (B). Regression slopes show that any WUE_i gained at peak severe drought stress was maintained after irrigation.

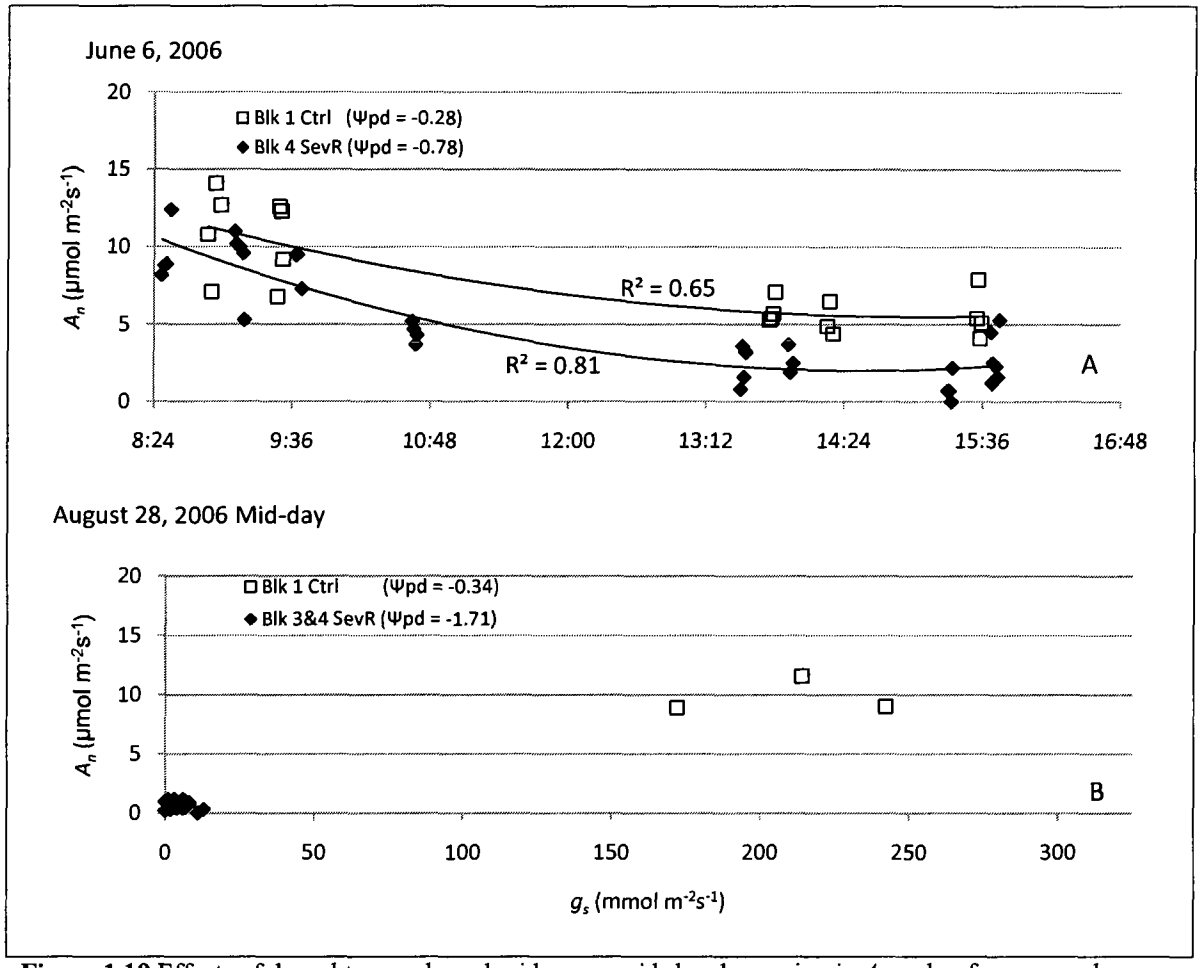


Figure 1.19 Effects of drought on early and midseason mid-day depression in A_n and g_s for green ash treatment Blocks 3 and 4 (SevR) and Block 1 (Ctrl). Despite reduced growth in Blocks 3 and 4, early season diurnal changes in A_n and g_s were equivalent to control Block 1 (A). At peak drought stress on August 8, mid-day depression of A_n and g_s in Blocks 3 and 4 represented a condition of maximum resistance to water loss (B).

Block 7 (ModNR)

The response of A_n and g_s to the extended moderate drought stress in Block 7 was similar to the other drought stressed blocks despite a unique relationship with Block 1 (Ctrl). Although Block 7 did not receive any recovery irrigation, Ψ_{pd} did not drop to the levels measured in the other treatments. These higher Ψ_{pd} levels were not a result of low g_s and the subsequent water conservation. Therefore, another water source not controlled by the

irrigation system explains the moderate drought stress in Block 7 throughout the 2006 season. Morning A_n and g_s from May 29, 2006 and August 17 and 30, 2006 in Block 7 have a correlation that was nearly identical to control Block 1(Figure 1.20). Although actual Block 7 A_n and g_s on all days was lower than Block 1, the range of An in both a drought stressed and well watered block provides a complete data set that represents the classic curvilinear relationship associated with A_n and g_s The unique relationship between A_n and g_s is supported with two other characteristics shared by Block 7 and Block 1; an uncharacterized secondary water source and similar leaf relative water content (RWC). Average predawn leaf RWC for Block 7 and Block 1 were 91 percent and 97 percent respectively. The high predawn leaf RWC in Block 7 was 10 percent greater than any other drought stressed treatment block.

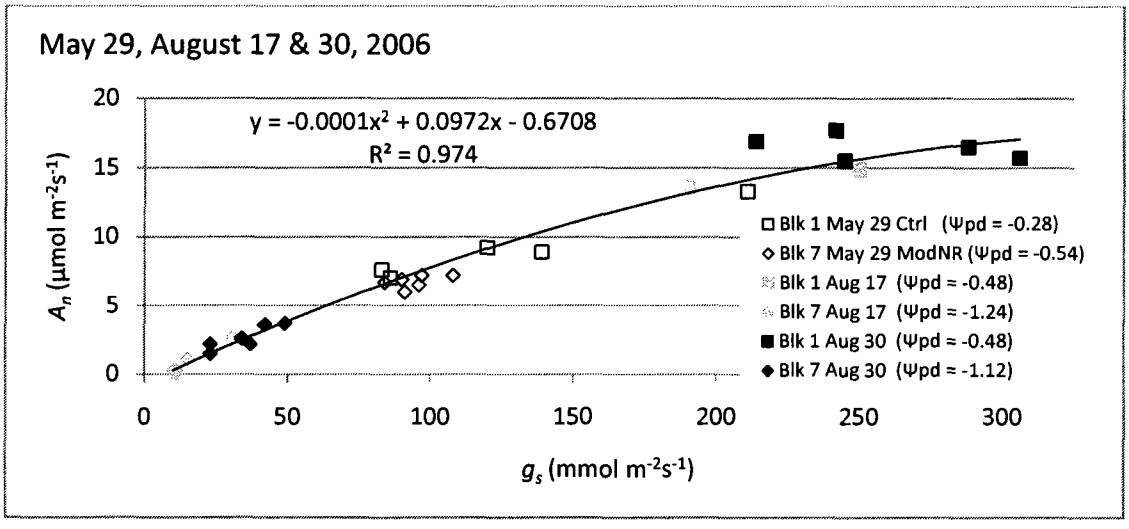


Figure 1.20 Effects of drought on A_n and g_s for green ash treatment Block 7 (ModNR) and Block 1 (Ctrl). Unlike plots for other blocks, measurements from both Blocks 1 and 7 plotted on the same regression line. Data for Block 7 represent morning measurements on May 29, 2006, August 17, 2006 and August 30, 2006. The lack of early season drought stress coupled with moderate seasonal drought stress resulted in WUE_i, represented by the regression line, equivalent to Block 1.

The length of drought imposed on Block 7 had a measurable effect on fall leaf senescence. Peak color change and complete leaf drop occurred in Block 7 trees approximately two weeks earlier than in Block 5. Color change and complete leaf drop in Block 1 occurred 10 days after Block 5.

Trunk Growth

Smaller leaf size, relative to the control block, as a result of early season drought stress, and reduction in A_n, as a result of prolonged drought stress, affected trunk growth (Table 1.7). Block 1 (Ctrl) had significantly more growth than all drought stress treatments. Within the treatments, only growth in Blocks 3 and 4 (SevR) were significantly different (P=0.003). Although Block 9 had the lowest average growth and several trees within the blocked accumulated no growth, variability within the block was also the greatest.

	Trunk Initial Diameter (cm)	Trunk Final Diameter (cm)	Cross Sectional Increase (cm^2)	2006 Increase (%)
Block 1 (Ctrl)	16.53	17.54	26.92 ± 5.45	12.7
Block 2 (SevR)	10.39	10.69	5.04 ± 3.12	6.1
Block 3 (SevR)	11.55	11.71	2.91 ± 0.93	2.8
Block 4 (SevR)	11.45	11.75	5.39 ± 1.44	5.8
Block 5 (SevR)	10.96	11.19	4.05 ± 1.79	4.1
Block 7 (ModNR)	9.83	10.11	4.53 ± 2.72	5.7
Block 9 (ModR)	9.68	9.80	2.24 ± 3.45	2.3

Table 1.7 Effect of drought stress treatment on 2006 green ash trunk growth. Block 9 had the lowest early season Ψ_{pd}, the smallest trunk size increase and the greatest variability (n=7 trees per treatment).

Lesser trunk growth in the drought stress treatments, relative to control trees, effectively summarized the cumulative effect of drought stress through 2006.

Fundamental to this investigation is the intuitive concept that plants respond to their environmental conditions in ways that adapt and optimize their growth and ongoing biochemical and physical processes within the limits of their genome. (Schymanski et al. 2007) The green ash trees at ARDEC have a limited ability to respond to short-term drought stress that falls within the range identified by Abrams et al. (1990) and Shumway et al. (1991) who looked at five progenies of green ash along a longitudinal gradient. The green ash response to drought stress was consistent with the general theoretical and empirical understanding of plant reaction to drought stress. Early season drought stress lessened leaf growth and the smaller leaves were maintained throughout the growing season due to the determinant nature of green ash. Cessation in growth due to lower turgor pressures was expected with only a 0.30 MPa drop in water potentials (Shumway et. al. 1991; Hsiao 2000; Passioura and Munns 2000; Lovisolo et. al 2002). The data collected on May 21 and 28, 2006 show that a record dry winter was sufficient to drop Ψ_{pd} in all blocks, except Block 7 (ModNR), by 0.5 MPa when compared to Block 1(Ctrl).

Under lower Ψ_{pd}, malate and manitol were shown to accumulate in leaves of *Fraxinus excelsior* (Guicherd et al. 1997). That osmotic adjustment helped maintain stomatal conductance and prevent wilting down to a Ψ_{pd} of –6.0 MPa. However, assuming that green ash has similar osmotic regulation, the observed reduction in growth exposed the limits that osmotic adjustment has on maintaining sufficient cell turgor for leaf expansion. When considered in context of the whole tree and its immediate environment, reduced growth under drought stress was an effective response to early season drought stress. By definition, with all other conditions constant, smaller leaves had the effect of lowering overall transpiration and reducing the demand on the remaining available soil

moisture reservoir. The reduced canopy, fixed early in the season, must be considered when evaluating the ability of the leaf to conduct photosynthesis, control stomatal aperture and further reduce transpiration as drought stress increased to the moderate and severe levels reached in this study. The reduced growth became a hedge against further stress to levels that would induce permanent injury through leaf damage or even embolism (Braatne et.al. 1992, Cochard et al., 2002, Sperry et al., 2002).

Findley (1999), in 1998, attempted to assess the effects of drought stress on green ash but never achieved Ψ_{pd} lower than -1.1 MPa during the growing season. The data collected during August, 1998 showed a decline in Ψ_{pd} due to irrigation treatment sometime after August 6 but the magnitude of that decline was only 0.4 MPa lower than the 160% control. In contrast to past work at ARDEC, tree water status and leaf gas exchange in both 2005 and 2006 definitively established the presence of moderate and severe drought stress that was responsible for reduction in growth followed by depression of photosynthesis throughout the growing season. The magnitude of drought stress in the ARDEC established trees also provided a realistic lower threshold of drought stress that one might find in a managed landscape.

As soils at the site dried, resulting in decreasing Ψ_{pd} with time, the variability in declining Ψ_{pd} between trees in the same drought stressed treatment block increased dramatically. Jones (1997) warns that Ψ_{pd} is difficult to use as an indicator of stress when variability in individual trees or within treatments exceeds that between treatments. Although there was variation between trees within each treatment block (Figures 1.11, 1.14), all trees

within the block tracked the declines in parallel. In addition, the leaf gas exchange data for each block show similar A_n and g_s response to drought stress regardless of the tree specific Ψ_{pd}. With gradual declines in soil moisture and increasing diurnal and seasonal VPD associated with an arid environment, Ψ_{pd} in conjunction with periodic RWC was adequate for assessing drought stress in green ash at ARDEC. Based on the response of both A_n and g_s to drought stress, the threshold Ψ_{pd} that induced stomatal control was approximately –1.1 MPa. Ψ_{pd} below -1.1 MPa resulted in lower g_s and it was that response that helped restrict further water loss and maintain hydration status within the constraints of the tree specific environmental conditions. In all treatment blocks, Ψ_{pd} for individual trees that dropped below approximately –1.5 MPa was sufficient to reduce A_n and g_s. For all trees reaching the -1.5 MPa Ψ_{pd} threshold, leaf water potentials (Ψ_l) measured in conjunction with gas exchange ranged between -2.06 and -4.13 MPa with A_n reduced to between zero and 5 umol $m^{-2}s^{-1}$. That range was consistent with other work on green ash and related species that also showed a limited rebound of A_n and g_s within 24 hours of irrigation (Abrams et al., 1990; Angelopoulos et al., 1996; Carlier et al., 1992; Davies and Kozlowski, 1977).

1.4) Summary

These studies provided a range for Ψ_{pd}, A_n and g_s in green ash under drought stress and confirmed several important characteristics. The timing of drought stress is critical and early season stress restricted leaf growth and canopy size. In the determinant green ash, smaller leaf size cannot be overcome by new growth if drought stress is ended. Therefore, early season drought stress establishes limits to total carbon assimilation in

any given year. If drought stress is extended and becomes more severe, g_s is dramatically reduced and limits further Ψ_{pd} declines in established trees. The response of A_n and g_s to drought stress is definitive but it does not correlate well with Ψ_{pd}. Instead, A_n and g_s are triggered by Ψ_{pd} thresholds. Recovery of Ψ_{pd} after stress is not necessarily followed by complete recovery in g_s and A_n. However continued stomatal control of water loss provides optimum WUE_i and allows for the maintenance of minimum levels of A_n throughout the growing season.

Chapter 2 Non-Structural Carbohydrates

2.1) Introduction and Literature Review

Throughout the Front Range of Colorado, municipalities have developed urban forest management plans that focus on preserving the health of landscape trees and promoting an increase in the canopy cover. In addition, growth of the urban forest is seen as an offset to CO_2 emissions through carbon sequestration. As recently as 2004, these same municipalities voiced concern for the amount of water available for landscape irrigation with some cities restricting water use. Consequently, tree decline and even death has occurred. Anecdotally, the cause of this decline is generally attributed to drought stress but there are no specific studies that support that conclusion. In green ash, a species that constitutes up to 15 percent of the urban landscape, results of reduced growth and A_n, as a result of drought stress, may have an indirect effect on long-term tree health by reducing carbohydrate reserves (see Chapter 1).

There are no studies of green ash trees that measured changes in total non-structural carbohydrates (TNC) as a result of drought stress. By flooding green ash trees Gravatt and Kirby (1998) achieved a comparable condition of depressed A_n and g_s. Overall root growth was reduced while root starch concentrations were the same for control trees and trees with reduced A_n. Work conducted on other species lends a little insight into the changes in root TNC reserves due to stress. Liu and Tyree (1997) analyzed TNC concentrations in course and fine roots of healthy sugar maples trees and declining nutrient deficient trees with reduced canopies. Their results show that there was no difference in TNC concentrations between the healthy and nutrient stressed trees even

though the nutrient deficient vegetation had depressed A_n. Kosola et al. (2001) found similar results in poplars that were defoliated to mimic extreme herbivory. They found that by November, after a second flush of leaves occurred, there were no differences in root TNC concentrations between controls and defoliated trees. Coarse roots (> 2 mm diameter) had the greatest concentration. These studies support the idea that concentrations of TNC may remain the same in stressed trees but the lack of carbohydrate assimilation associated with stress will inhibit growth.

Not all research indicates maintenance of TNC concentration in stressed trees. Studying red spruce under drought conditions using potted trees, Amundson et al. (1993) found that starch concentrations in the fine roots (<3.0 mm) of well watered trees was significantly greater than concentrations in drought stressed trees. Samples were collected in November after recovery from drought and the results suggest that the reduced carbohydrate assimilation in the stressed trees hindered the development of fine roots. Foliar TNC concentrations were maintained at the expense of the roots. Coarse roots have the highest concentrations of stored carbohydrates (Marshall and Waring, 1985) so fine roots may not have been the appropriate indicators of a drought effect. However, Kolb and McCormick (1991) showed that small roots (<10 mm in diameter) of sugar maple had the greatest concentrations of non-structural carbohydrates.

The question addressed in this study was whether TNC reserves of green ash were altered by drought. In green ash, early bud set established a definitive limit to seasonal growth (see Chapter 1). The accumulation of carbohydrate stores during the remainder of the

growing season became a function of A_n. Non-stressed trees with higher A_n might be expected to store greater amounts of TNC. Higher concentrations of TNC would then be available for initial growth in the following year. In contrast, trees under drought stress experienced reduced vegetative growth prior to bud set which resulted in less leaf area for photosynthesis. In addition, drought stress that extended throughout the growing season further reduced g_s and A_n.

By measuring TNC in roots, trunks and first year stems, this study tested the hypothesis that the combined effect of reduced leaf area and lowered A_n per unit leaf area would result in reduced concentrations of TNC. Reduced concentrations of TNC could adversely affect bud break and limit initial stem growth the following year even if drought stress was eliminated prior to bud break. Therefore, drought stress in one year might carry over to subsequent years even if the drought did not.

2.2) Methods

Stem, trunk core and root samples were collected from trees representing four blocks receiving three different irrigation treatments. Block 1 was a fully hydrated control (Ctrl). Blocks 4 and 5 received severe drought stress with recovery irrigation (SevR) and Block 7 received moderate drought stress without recovery irrigation (ModNR)(Table 1.3). Samples were collected on three different dates, Sept 8, Sept 22 and Oct 6, 2006, to assess overall changes in the storage of TNC as a function of progression toward dormancy.

Root samples were collected from shallow hand dug pits near the center of designated blocks and equidistant to the four nearest trees. Pits were large enough to collect a sufficient quantity of similarly sized roots for analysis. All roots collected were near 10 mm or slightly smaller in diameter. One composite root sample from each of the four blocks was collected on each sampling date.

Stem samples were collected, after complete leaf drop, as entire branchlets corresponding to the current year's growth and stored on ice. As stem growth was affected by treatment (Chapter 1), stem diameters varied between treatments. For each sampling date, there were six Ctrl samples from two different trees, eight SevR samples from six different trees and three ModNR samples from three different trees. Both root and stem samples were processed in their entirety.

After bark was removed down to the cambium, trunk cores from selected trees were collected using a 4mm diameter increment borer to a depth of 10 mm. On each date trunk cores were sampled from a different tree in each treatment block. The different trees corresponded to those used for stem samples. Once all samples were weighed, freeze dried and weighed again, they were ground in a small rotating blade mill for analysis.

The methods for TNC analysis were based on Haissig and Dickson (1979), and Hansen and Møller (1975) with variations to the methods as described by Rose et al. (1991), Grantz and Wang (2000), Laurentin and Edwards (2003), Chow and Landhäusser

(2004), and Olano et al. (2006). All results were reported as a glucose equivalent. Samples were analyzed for sugars using an anthrone reaction in a spectrophotometric microplate analysis. The anthrone colorimetric method cannot distinguish between sugars of increasing complexity such as fructose (monomer) and sucrose (dimer). Prior to analysis, it was unknown whether the TNC of green ash contained a large percentage of more complex sugars. In white ash and elm raffinose and stachyose may be as prevalent as sucrose (Zimmerman, 1957). However, in 19 other species raffinose and stachyose concentrations ranged from a trace to a maximum of 5 percent (Zimmerman, 1957, Hoch et al., 2003). To estimate the total glucose equivalent by determining the ratios of glucose, fructose (monomers), sucrose (dimer) and raffinose (trimer), selected samples were reacted with a 1-(trimethysilyl) imidazole – pyridine mixture and analyzed for relative abundance using gas chromatography-mass spectrometry (GCMS).

Starch content was determined by first reducing the starch to sugar through enzymatic hydrolosis and then analyzing the digestion product using a glucose oxidase/peroxidase (PGO) reaction in a spectrophotometric microplate analysis. The PGO reaction replaced the anthrone reaction because of interference from residual enzymes in the reduction process. A detailed description of the analytical method is provided in Appendix B. Differences in mean TNC concentrations, between treatments and tissue type, were determined using Student T-Tests at the 0.05 significance level.

2.3) Results and Discussion

GCMS results (not shown) identified glucose and fructose as the primary sugars in the selected root, trunk core, and stem samples. Glucose and fructose comprised slightly more than 90 percent of sugars, sucrose accounted for approximately 9 percent and raffinose accounted for less than 1 percent.

There was no effect of irrigation treatment on sugar or starch concentrations for the three dates that bracket the fall leaf drop and there was no trend of changing sugar or starch concentration as the season progressed (Figure 2.1). For the three sample types, there were no statistically significant differences in sugar or starch concentration between trees within a treatment, between treatment blocks, or between sampling dates. Roots stored the highest concentrations of sugar and starch, followed by stems.

The results of this study are similar to the conclusions of Gravatt and Kirby (1998) and Liu and Tyree (1997) who found no difference in TNC concentrations between trees undergoing drought stress and fully hydrated trees. The green ash trees in this study were subjected to drought stress for a much longer period than in other studies, but the effect of the prolonged drought stress was reduced growth rather than a reduction in concentration of stored TNC. Overall reduced growth meant less TNC on a per tree basis but the metabolism of new tissue was able to maintain TNC concentrations at levels similar to the control on a dry mass basis.

2.4) Summary

Roots of green ash have the highest concentrations of TNC and those concentrations are not altered by drought stress severity or duration. By maintaining consistent concentrations of sugar and starch, male green ash trees limited their response to drought stress by restricting leaf size. The lesser leaf size due to drought stress is significant and the resulting decrease in biomass is a direct consequence of reduced A_n. However, a mechanism exists that balances the reduction in biomass with consistent concentrations of TNC.

Green ash trees are ring porous and root storage of TNC provides the necessary energy for early season production of large xylem vessels. Growth in any one year is influenced by growth and bud set in the previous year. A strategy that restricts growth but maintains relative TNC concentrations provides an optimal carbohydrate economy. Drought affected growth in green ash did not produce a whole tree carbohydrate deficit that would dramatically hinder growth in subsequent years.

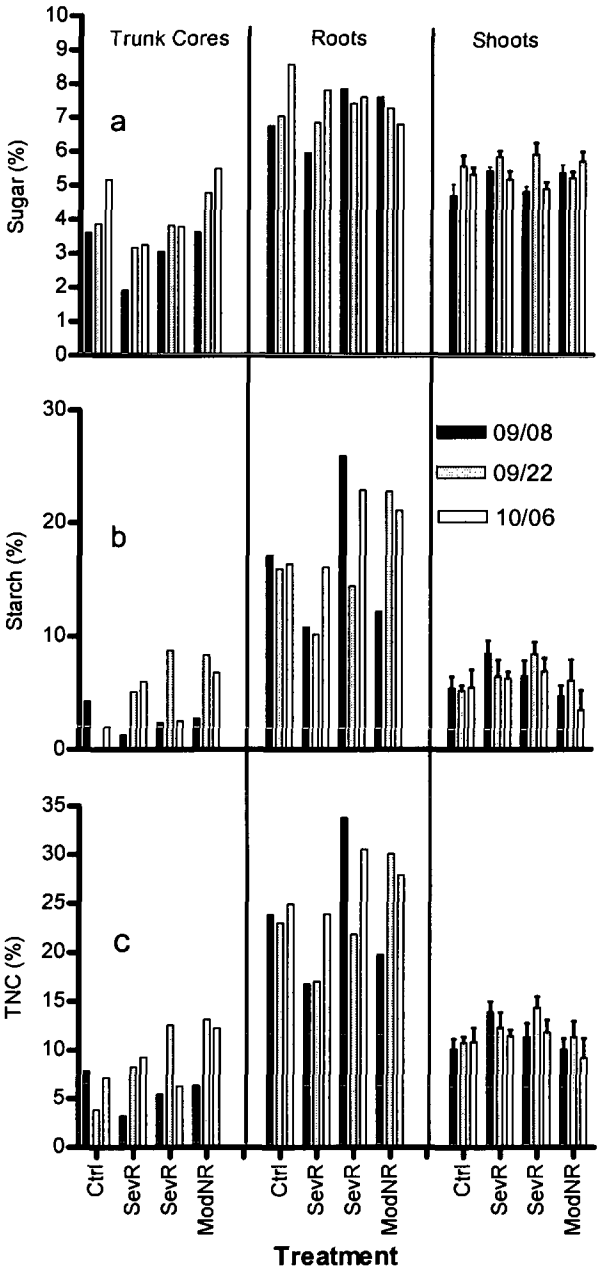


Figure 2.1 Effects of irrigation treatments on the concentration of sugars (a), starch (b), and total non-structural carbohydrates (c) in stems, trunks, and roots of green ash. Ctrl represents the fully hydrated control (Block 1). SevR represents a treatment (Blocks 4 and 5) which received severe drought stress followed by recovery irrigation. ModNR represents a treatment (Block 7) which received moderate drought stress without recovery irrigation. Concentrations of sugar, starch and TNC are percent dry weight of glucose equivalent. Error bars on stem columns represent one standard deviation. Sample size was limited to one sample per treatment per date for trunk cores and roots.

Chapter 3 Cold Hardiness

3.1) Introduction and Literature Review

A reduction in cold hardiness or a degradation of cell membrane integrity as a direct result of drought stress can have negative consequences on green ash tree health. However, landscape management strategies, such as late season irrigation may have a moderating effect on seasonal drought so that winter dormancy is not compromised.

The progression toward cold hardiness in woody plants has been well researched (Banuelos et al. 2008; Gusta et al., 2005; Kalberer et al., 2006; Li et al., 2004; Mahajan and Tuteja; 2005, Tomashow, 1999; Wisniewski et al., 2003). There is a considerable body of evidence that drought stress at or near the end of the growing season can induce bud set and early dormancy which in turn increases cold hardiness (Kozlowski and Pallardy, 2002). More specifically, the idea of drought stress affecting the process of dormancy in woody plants has been proven in controlled situations. Douglas fir (*Pseudotsuga menziesii* Mirb.) seedlings exposed to drought stress of –0.5 to –1.0 MPa during the growing season showed increased cold hardiness to –20.25 °C (Blake et al., 1979). In these examples, drought stress helped to induce bud set but a further understanding of the mechanisms for improved cold hardiness was not explored. Alternatively, Olsen et al. (1997) showed that apical bud set is a response to photoperiod and not tied to drought stress during the normal seasonal progression. Drought stress induced cold hardiness in redosier dogwood (*Cornus sericea* L.)(Chen et al., 1977). Chen showed that the drought stress was able to increase the ability to both avoid and tolerate tissue ice. Findley's (1999) work at ARDEC, when green ash trees had been

established for three years, was conducted within the same range of water potentials and found no effect of drought stress on cold hardiness.

It is possible that the apparent similarities between metabolic changes associated with protection from either drought or cold stress may be closely linked to the limited number of adaptive strategies available to plants. For example, a detailed study of stress responses in cultured aspen (*Populus tremuloides* Michx.) roots and stems (Pelah et al., 1995, 1997) showed that drought and cold stress induced the expression of stress response proteins and sucrose synthase along with increased sucrose levels and decreased glucose levels. Cox and Stushnoff (2001) showed that aspen stems exhibit temperature related shifts in soluble carbohydrates during cold acclimation. In aspen, the shifts in sugar may have allowed buds to avoid ice nucleation. Cox and Stushnoff (2001) found that the shift was toward the trimer, raffinose. While the Cox and Stushnoff (2001) study did not address the effects of drought stress it did show that shifts in sugar chemistry were an important factor in cold hardiness. Based on the importance of sufficient sugar reserves, a severe reduction in carbohydrate assimilation, through extended drought stress, potentially reduces the ability of trees to increase or shift sugar levels during cold acclimation (Gusta et al., 2005).

Research within the ash genus is less detailed, but work by Guicherd et al. (1997) described the drought stress response. They found that under severe drought stress European ash produced the organic acid malate and sugar alcohol manitol but not sucrose. As osmoticum, these compounds are derived from the modification of sugars

and starches. The influence of carbohydrates is a recurring theme in these studies and suggests that any environmental stress that would alter photosynthesis would also have some impact on cold hardiness. Of course, the study of drought on cold hardiness may be moot if the effect on cold hardiness occurs only at temperatures well below anything experienced in the region. Green ash grows in a geographic range that includes cold hardiness zone two and reaches minimum temperatures of -50 °C (Wright, 1965). However, green ash landscape use in Colorado exists in regions that experience a minimum temperature of -31 °C. To understand changes in cold hardiness at temperatures insufficient to cause death, membrane leakage measurements was used to provide an indicator of cell damage that is still potentially injurious (Bajji et.al. 2001).

In my 2006 study, cold hardiness and membrane leakage were measured in green ash trees after severe and extended drought stress (Ψ_{pd} = -1.36 to -2.56) followed by late season irrigation. Considering the opposing factors of drought stress and late season irrigation, the fundamental question was whether the resulting reduction in photosynthesis affected the green ash metabolism sufficiently to inhibit cold acclimation.

3.2) Methods

Summary of Drought Stress

Green ash trees at ARDEC were subjected to three levels of drought stress. Block 1 was a fully hydrated control with a minimum average block Ψ_{pd} of -0.72 MPa (Ctrl). Block 5 received severe drought stress with recovery irrigation and achieved a minimum average block Ψ_{pd} of -2.37 MPa (SevR). Block 7 received moderate drought stress without

recovery irrigation and achieved a minimum average block Ψ_{pd} of -1.36 (ModNR). Drought conditions began in the fall of 2005 and continued through 2006. Cumulative ET_o demand for the year exceeded 800 mm (Figure 3.1). Irrigation of the Ctrl began in May and continued at a rate slightly higher than ET_o so that by the end of September the cumulative irrigation was equal to ET_o. Block 5 (SevR) received only natural precipitation from October, 2005 until recovery irrigation was initiated on August 24, 2006 and again on September 30, 2006. Block 7 (ModNR) received only natural precipitation from October, 2005 until site-wide irrigation on September 30, 2006, two weeks after fall leaf abscission. The late season irrigation of Block 7did not offset the drying conditions indicated by the cumulative ET_o. However, based on Ψ_{pd} increases after irrigation in the SevR blocks, the late season irrigation was sufficient to relieve the drought stress.

Leaf relative water content (RWC) in SevR and ModNR treatments was dramatically different (see Chapter 1). On August 17, 2006, predawn RWC showed that Block 5 (SevR) ranged between 74 and 87 percent. However, RWC in Block 7 (ModNR) ranged between 93 and 96 percent which was similar to the 98 percent RWC of the Ctrl. Based on both RWC and Ψ_{pd}, this study achieved drought stress in the SevR treatment that was comparable to levels achieved by Pelah et al., (1995,1997) and Guicherd et al. (1997). Similar to European ash (Guicherd et al., 1997) the immediate depression of A_n as a result of drought stress was reversed upon application of recovery irrigation.

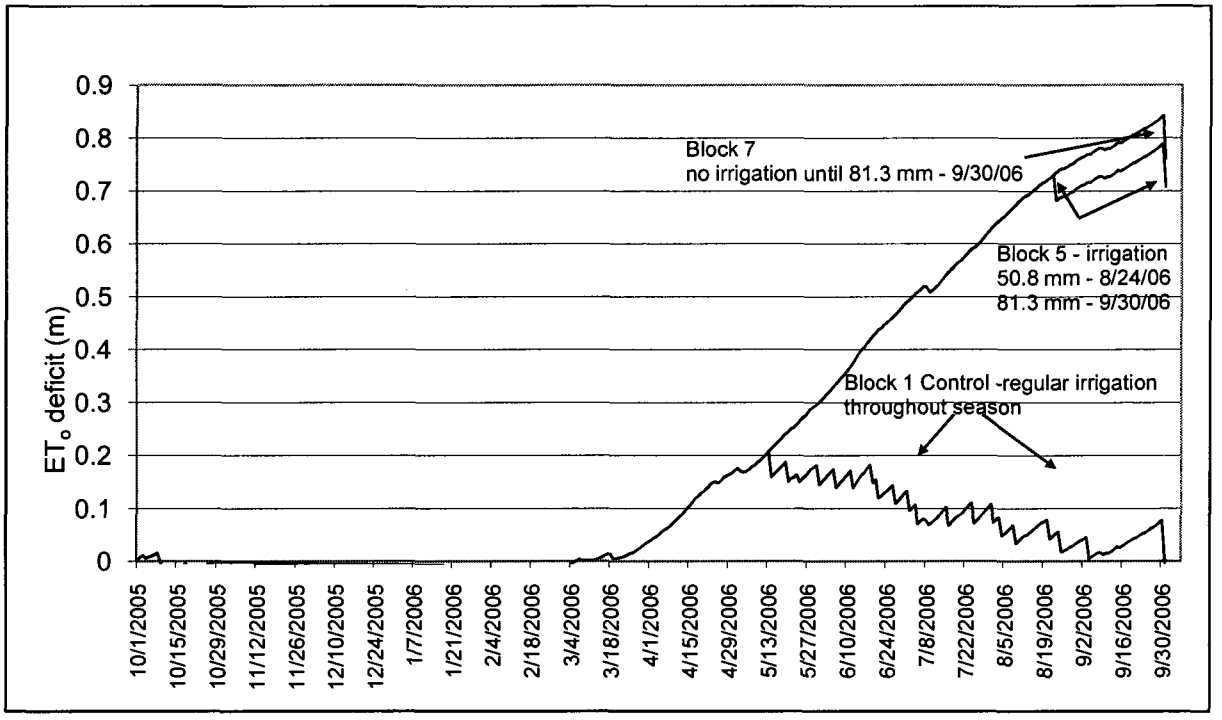


Figure 3.1 The effect of drought on ET_0 deficit in green ash trees at ARDEC. The rising line represents the cumulative ET_o as calculated for grass. The line diverges on May 13, 2006 with the initiation of irrigation in Block 1(Ctrl). Regular irrigation in Block 1 was sufficient to reduce the deficit to zero by the end of September, 2006. The line diverges again on August 23, 2006 when irrigation was initiated for recovery in treatment Block 5(SevR). Irrigation of Block 7 (ModNR) occurred on September 30, 2006, two weeks after fall leaf abscission.

Sampling and Freezing

On December 18, 2006 current year stems from trees in the Ctrl, SevR and ModNR treatments were collected for freezing to a temperature of –30 °C. Preliminary results of that procedure indicated that drought stress had affected vegetative bud cold hardiness. To confirm those results, current year stems from eight trees in Blocks 1 (Ctrl), 5 (SevR) and 7 (ModNR) were collected on January 26, 2007. There was no loss of cold hardiness between December and January due to consistently cold temperatures during the period between sample collections. Terminal branchlets from the canopy between 6.7 and 4.9 meters above the ground were collected from scaffold branches. Only branchlets that received full sun for the majority of the day the previous growing season were collected

for testing. Seventy-five stems from each tree were collected to supply 10 replicates for each targeted temperature down to a maximum of –50 °C. Samples were stored on ice until they were prepared for freezing.

Using a Tenney Junior bench top programmable temperature chamber, stems were wrapped in wet paper towels and sealed in plastic bags so that they could be rapidly removed when they reached their predetermined temperature end point. Starting at 0 °C the temperature was dropped at the rate of 2.5 °C per hour to –25 °C and then held for one hour. At that point the first set of stems was removed and stored at room temperature. The temperature drop continued at 2.5 °C per hour to -30 °C and then held for one hour at which time the second set of stems was removed. These freeze steps continued in five-degree increments until the final stems had been exposed to –50 °C. All stems were stored in the dark at room temperature for six days prior to testing for membrane leakage.

Membrane Leakage

After the freezing regime, terminal buds from all branchlets were excised and split along the widest axis so that interior surfaces were exposed. The cut buds were visually assessed for freeze damage and placed in 100 cell trays for measurement of solution conductivity associated with membrane leakage (σ) using a Neogen Asac 1000. Two milliliters of nanopure water were added to each cell. Measurements were immediately initiated and continued at the frequency of every 10 minutes for 8 hours. This duration accounted for the biphasic kinetics by measuring the initial rapid apoplastic leakage and

continued into the slower more linear symplastic leakage (Kocheva et al., 2005; Prasil and Zámečnik, 1990; Leopold et al., 1981). Upon completion the trays were placed in a –80 °C freezer until all living tissue was killed (Aniśko and Lindstrom, 1995). The trays were then thawed and measured to determine maximum leakage (σ_{max}) defined as the specific conductance of the solution three hours after the final thaw. The buds were then dried for approximately 12 hours at 70 °C and weighed to an accuracy of 0.1 mg. Raw data from the different runs was first calibrated using a 10-point sodium chloride standard included in each tray and then converted to represent μScm^{-1}. Finally, the calibrated and converted data was adjusted to $\mu Scm^{-1}mg^{-1}$ through division by dry weight in milligrams.

In order to assess cell membrane damage from freezing, leakage at time t (σ_t) was calculated for three and four hours as follows:

$$\sigma_t = \frac{C_t}{m}$$

where C_t is the specific conductance (μScm^{-1}) at time t and divided by the dry weight of the sample m (mg) so that the results are standardized per milligram of sample.

Maximum leakage σ_{max} is defined as:

$$\sigma_{max} = \frac{C_{max}}{m}$$

where C_{max} is the maximum leakage per milligram of sample after the sample had been frozen to –80 °C and then allowed to thaw for three hours.

Relative leakage is defined as:

$$\sigma_{rel} = \frac{\sigma_t}{\sigma_{max}} * 100$$

Leakage measurements are dependent on time. Symplastic leakage is expected to continue for 20 hours or longer (Prasil and Zámečnik 1990; Aniśko et al. 1995). However, as Kocheva et al. (2005) observed the leakage after four hours is more linear when compared to the first four hours. It follows that the relative effects of drought stress treatments and freezing temperature measured by membrane leakage within the first four hours are generally maintained for the duration of the leakage measurements. To assess the relationship between Ctrl, SevR and ModNR treatments σ_{rel} was calculated using σ_t at both three and four hours. Effects of treatment and tree on σ_3, σ_4 and σ_{rel} were determined using general linear analysis of variance model (SAS Institute, 2008). The relationship between Ψ_{pd} and the 0 - 40 °C σ_{rel} was determined by assessing the coefficient of determination (R^2) produced from regressing Ψ_{pd} on σ_{rel} for the all trees included in the sampling.

3.3) Results and Discussion

The initial assessment of freezing damage consisted of visual inspection. Parenchyma became darkened in some samples at –45 °C. However, cambial phloem and xylem remained bright green indicating that there was no damage to –50 °C. The terminal buds in green ash are composed of an apical vegetative bud subtended by a pair of axillary flower buds. Flower buds on the samples collected varied in their stage of development. Some had obvious expanded pollen sacks while others were tightly packed. Of the 103 expanded flower buds, two were killed at –45 °C and two were killed at –50 °C. Those four buds were black and necrotic and were collected from the SevR treatment (5D, 5C).

All other buds, either vegetative or flower, showed no signs of freezing damage. The lack of freeze damage at temperatures down to -50 °C did not allow for the determination of lethal temperature thresholds.

However, by reviewing the warmest and coldest temperatures tested, the greatest differences between σ were assessed. Membrane leakage (σ) and σ_{rel} curves for 0 and –50 °C in the SevR (Block 5) and ModNR (Block 6) treatments and the Ctrl (Block 1) showed high variability between trees (Figure 3.2). As expected, even after eight hours of measurements, leakage continued to increase. However, the slope was much shallower and more linear after four hours. There was an increase in σ for the –50 °C samples in the Ctrl treatment. However, for the SevR and ModNR treatments, there was no consistent trend in σ between the zero and -50°C temperatures.

If there were a drought stress effect on membrane leakage, one would expect to see consistently higher σ_{rel} in the SevR and ModNR treatments. In addition, if cold hardiness was adversely affected one would expect to see greater σ_{rel} in samples exposed to –50 °C. However, there were no significant differences between treatments. Differences in σ_{rel} between trees within drought stress treatments were as large as differences between treatments suggesting that there may be a tree effect rather than a treatment effect.

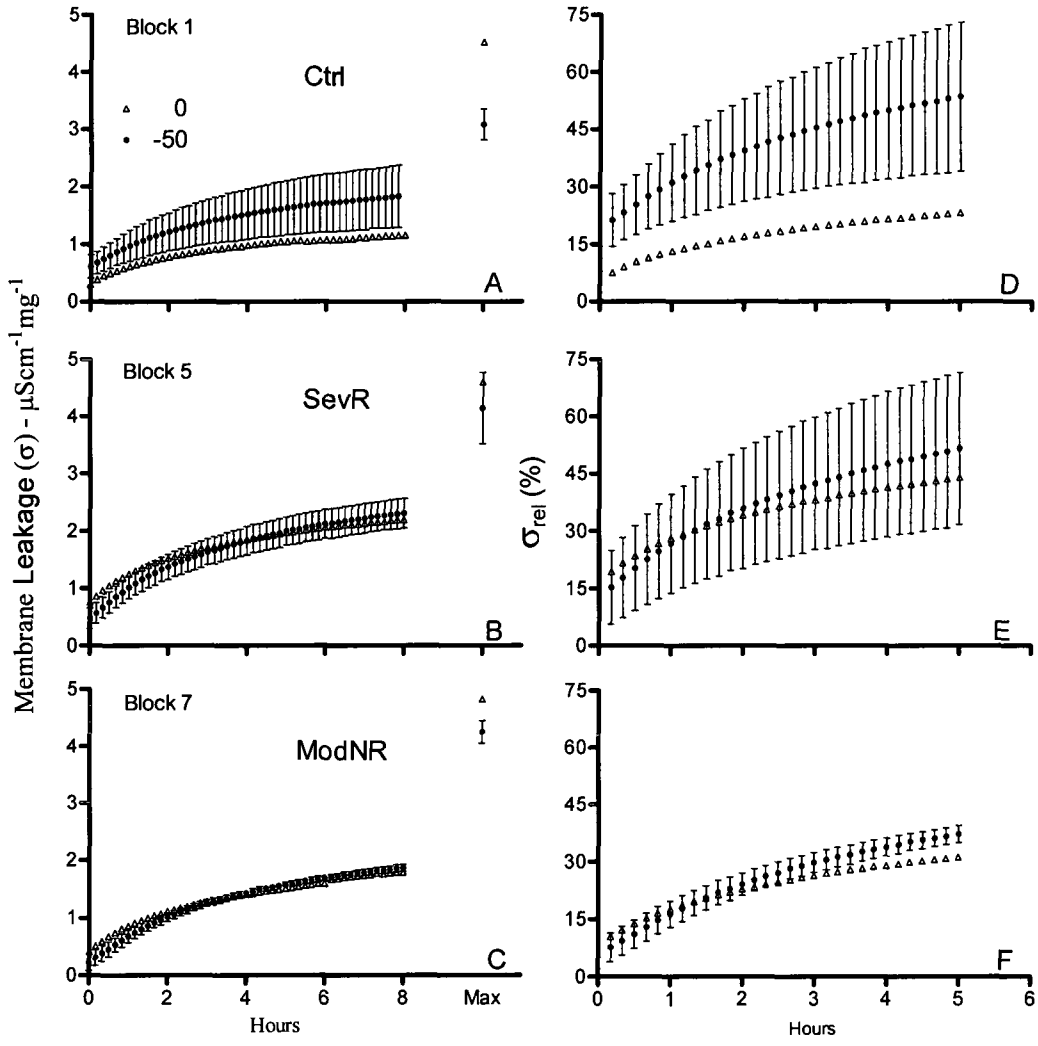


Figure 3.2 Green ash bud membrane leakage (σ) and percent of maximum leakage (σ_{rel}) for Ctrl, SevR and ModNR treatments at zero and -50 °C temperatures. The Ctrl treatment (A) shows σ in the buds subjected to -50 °C with a reverse relationship for σ_{max}. The SevR and ModNR treatments showed no discernable trend in σ or σ_{max} with temperature. Differences were largely tree specific rather than treatment specific. Max points represent σ_{max} for each tree sampled. The Ctrl treatment (D) shows greater σ_{rel} in the buds subjected to -50 °C. The SevR treatment (E) showed no consistent trend of greater σ_{rel} in the buds subjected to -50 °C. The ModNR treatment (F) showed steeper slopes for σ_{rel} at 4 hours in the buds subjected to -50 °C. Each curve is constructed from the average of 10 samples. Error bars on the -50 °C curves represent one standard deviation. Error bars were not added to the 0 °C curves to allow visual clarity. However, they were of similar magnitude to -50 °C.

The SAS General Linear Model was run on σ_3, σ_4 and σ_{rel} for treatment (tree), temperature and treatment-temperature interaction (Table 3.1). The significance of percent maximum leakage for tree, temperature and the tree-temperature interaction suggests that it is the best indicator of stress effects on cold hardiness.

Drought Stress Treatment	Ctrl(1E,1F), SevR(5D,5E,5G), ModNR(7B,7G,7H)		
Temperatures	0 -25 -30 -35 -40 -45 -50 ^{0}C		
	Probability > F Value		
	3-Hour Rate	**4-Hour Rate**	**% Maximum Leakage**
Temperature	0.3131	0.1108	**<.0001**
Tree	**<.0001**	**<.0001**	**<.0001**
Temperature * Tree	**<.0001**	**<.0001**	**<.0001**

Table 3.1 Results of SAS General Linear Model comparing drought stress and temperature treatment effects as determined by membrane leakage (σ) at 3-hours (σ_3), 4-hours (σ_4) and as percent of maximum leakage (σ_{rel}). Drought stress treatments were defined by individual trees so that differences within treatments could be distinguished from differences between treatments. The probability that means are statistically significant reflects a 0.05 significance level.

A more detailed review of σ_{rel} by temperature for individual trees within each treatment showed that there was no significant difference between temperatures 0 to –40 °C. However, the -50 °C σ_{rel} was significantly different from other temperatures in all trees and the -45 °C σ_{rel} was significantly different than other temperatures in four of the eight trees. Based on the differences in σ_{rel} between different temperatures for individual trees, σ_{rel} for the temperature ranges 0 to -40 °C were pooled and compared to the temperature specific results for -45 and -50 °C (Figure 3.3).

The separation of σ_{rel} into the three temperature categories clearly shows that the affect of the colder temperatures, -45 and -50 °C was tree specific. At these lower temperatures,

the control trees from Block 1 (1E and 1F) were just as likely to see increased σ_{rel} as trees subjected to either the SevR (5D, 5E, 5G) or ModNR (7B, 7G, 7H) drought stress treatments. However, data from the range 0 to -40 °C show a significant trend associated with drought stress level.

As measured by progressively lower Ψ_{pd}, σ_{rel} at these sub lethal temperatures increased with drought stress ($R^2 = 0.70$ P=0.009). The differences in σ_{rel}, while insignificant between the Ctrl (1E, 1F), are significant from the Ctrl for trees receiving the SevR (5D, 5E, 5G) treatment and ModNR (7B, 7G, 7H) treatment. This supports the conclusion that a drought stress threshold greater than -1.5 MPa is needed for cold treatment at sub-lethal temperatures to affect membrane integrity.

3.4) Summary

Research showing early induction of bud set as a primary response to drought stress does not directly apply to the green ash in this study. Terminal and axial buds were established by mid-June. Consequently, progressive drought stress through the summer had no effect on bud set. There is a clear advantage for trees to induce bud set and initiate cold hardiness under normal summer conditions. In Colorado, peak summer conditions with high VPD can reduce Ψ_{pd} of non-stressed trees to levels shown to induce bud set (Blake et al., 1979). Therefore, an adaptation by species to induce cold hardiness under normal late summer conditions would be a requisite trait. However, an induction and improvement in cold hardiness, because of drought conditions many months in advance of the need for cold hardiness, does not produce a clear advantage.

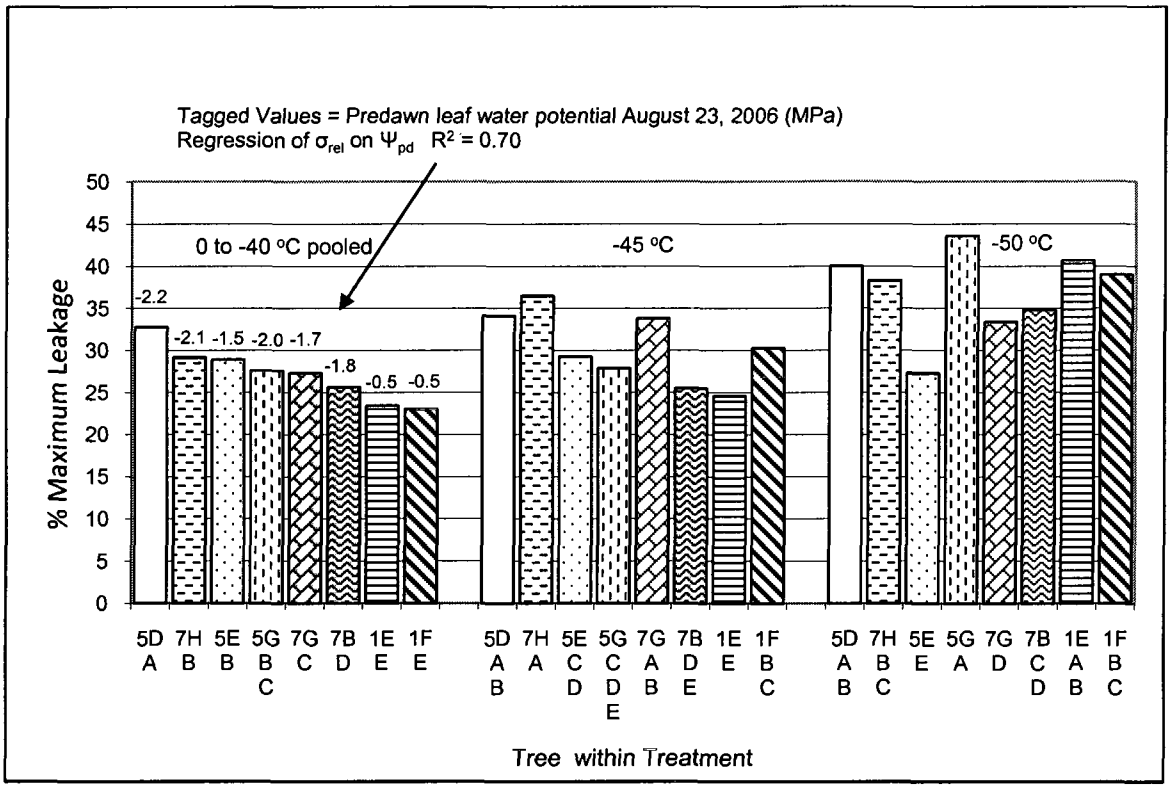


Figure 3.3 Tree wise comparisons of σ_{rel} for -45 °C, -50 °C, and pooled results of temperatures 0 to – 40 °C. Tagged values on the 0 to -40 °C group represent Ψ_{pd} on August 23, 2006. Although mean σ_{rel} for individual trees are not all significantly different, a regression of σ_{max} on Ψ_{pd} was significant with $R^2 = 0.70$ P=0.009. Column fill patterns remain the same for specific trees in each of the three temperature groupings. Trees with the same letter (within each temperature groupings) are not significantly different.

The overall effect of drought stress on the green ash in this experiment should be considered within the context of the irrigation schedule. At or near the peak of drought stress, the SevR treatment was irrigated to measure recovery response and may have negated any reduction in cold hardiness as a result of drought stress. Subsequently, the site wide irrigation on September 30, 2006 may have affected the ModNR in the same way. It is clear that under these conditions temperatures down to -50 °C were not lethal to the vegetative terminal buds but they did have a sub-lethal effect on cell membrane integrity. At temperatures between 0 and -40 °C drought stress affected σ_{rel} such that lower Ψ_{pd} produced higher cell membrane leakage.

While the late season irrigation in this study may not have allowed for a true measure of absolute drought stress on cold hardiness it did provide practical insight into the effects of drought stress within the framework of current landscape irrigation practices. Drought conditions that can limit spring growth and reduce A_n throughout the summer can be reversed with irrigation and do not have an immediate effect on cold hardiness. For a managed landscape subjected to periodic restrictions on water use, green ash is well adapted to withstand extended periods of drought stress and then recover without lasting effects when fall irrigation water is available.

Chapter 4 Measurements of Extreme Drought Stress Using a Weighing Lysimeter

4.1) Introduction and Literature Review

Journal articles on lysimeter use date back into the 1920s and include tree lysimeters as large as 29,000 kg (Fritschen et al., 1977). More recently, simple lysimeters have been used to estimate the equivalent of crop coefficients for individual plant species within complex landscapes (Abrams et al., 1990; Auge et al., 1998; Bauerle et al., 2002; Mingeau and Rousseau, 1994; Schuch and Burger, 1997; St. Hilaire et al., 2003). The technological advancements in scales and other weighing devices coupled with the availability of large plastic containers makes weighing lysimeters a simple and effective tool for accurately confirming the results of soil moisture depletion. The broad acceptance, diverse use in industry, easy construction from a variety of materials, and general familiarity associated with the design and implementation of these devices is the primary reason, with notable exceptions, (St. Hilaire et al., 2003) that details of lysimeter construction are seldom presented in the literature.

Measurements of daily ET, in container grown landscape plants, collected using some form of the tripod/container lysimeter system can be related to nursery operations that use containers for plant growth (St. Hilaire et al., 2003). Among the best systems is the double pot nursery method commonly known as pot-in-pot (PIP) (Ruter, 1996; Young and Backman, 1996; Martin and Ingram, 1999; Adrian et al., 1998). The pots are light, inexpensive, sturdy, and can be large enough to grow small trees. Using this system, St. Hilaire constructed a lysimeter that employed a tripod with a single overhead lifting point centered at the intersection of the legs. The tripod was used to lift the inner pot above the

outer pot that was buried into the ground. The inner lysimeter pots were then removed from the lifting device and placed on a common scale for weighing.

There is one important drawback to using a conventional tripod system with PIP tree lysimeters. Trees with trunk diameters of 31 mm can approach 3.5 meters in height. To lift trees that tall a tripod would have to exceed 4.5 meters in height to avoid damage to the canopy. Tripods of that height are difficult to maneuver and lose the benefits of a one-man operation.

To determine water use in young green ash trees during repeated dry down cycles, an innovative weighing system was built using a modified tripod with legs attached to a hexagonal frame that could be opened to accommodate tall trees. The design allowed for rapid and accurate weighing of multiple heavy lysimeters by a single researcher. The system was first tested in 2003 and 2004 on Freeman maples (*Acer x fremanii* 'Autumn Blaze') at the CSU Horticultural Research Farm. Then, in the spring of 1995, young green ash trees were planted at ARDEC using PIP techniques. The open tripod lysimeter was used to measure extreme drought stress ($\Psi_{pd} < 3.5$ MPa) on these potted trees and augment measurements of drought stress on established green ash trees at ARDEC.

4.2) Methods and Materials

Freeman maples

In May of 2003 #20 black plastic PIP outer socket pots were buried to handle depth on ten foot centers along an east-west alignment in native clay soil at the CSU Horticultural Research Farm. Matching inner pots were first coated with Spin Out® to limit root girdling. Nominal 25 mm diameter bare root Freeman maple trees were then planted in the inner pots using a local organic compost potting mix (Organix®) consisting of composted animal manure, pumice and finely ground wood chips. Osmocote® 14-14-14 was applied at the recommended rate. At six equidistant points around the perimeter of the upper lip of the inner pot two small holes were drilled through the plastic and a short length of cord was tied in a loop to act as a lifting point. Segments of 2x4 dimension lumber, cut to size, were placed in the bottom of the outer socket pot to allow the inner pot to move freely in and out. The trees were then set in place, staked to improve establishment and drip irrigated at a sufficient rate to exceed daily ET. A pressure chamber from PMS Instruments was used to determine leaf water potential.

Green Ash

In May of 2005 a system of PIP lysimeters, similar to the Freeman maples, was planted in an east-west alignment at ARDEC with 31 mm diameter bare root green ash trees. The primary difference was the use of native heavy clay soils rather than a potting mix. To facilitate lifting the heavier lysimeters, a ¾ inch plywood disk was cut to a diameter ½ inch greater than the diameter of the upper lip of the inner pot. Holes were drilled though the plywood to reduce weight and allow unrestricted drainage from the inner pot. The

discs were then coated with exterior urethane to prevent water damage. At six equidistant points around the perimeter of the plywood disc two small holes were drilled for the attachment of a lifting cord. These holes were in alignment with a single hole at the top lip of the inner pot such that a lifting cord attached to the plywood could pass through the upper pot and keep the inner pot and plywood base in a unitized configuration. The plywood disc provides a base that was required for pots filled with approximately 100kg of native soil so that the inner pots did not deform when lifting. The trees were set in place, staked to improve establishment, and drip irrigated at a sufficient rate to exceed daily ET.

Measurements of leaf gas exchange were collected using a Ciras II gas analyzer manufactured by PP Systems. Reference CO2 was set at 380 ppm. Leaves selected for measurement were analyzed in their natural orientation, usually toward full direct sunlight. A pressure chamber from PMS Instruments was used to determine leaf water potential.

Tripod Construction

One-inch square steel tube was welded into a hexagonal open ring tripod frame that was slightly larger than the diameter of the #20 PIP inner pots. At the junction of each side of the hexagon a 1/8 inch triangular steel plate was welded to the underside of the frame as a strengthening gusset and attachment point for eye bolts used to lift and suspend the #20 pots. A six-inch section of tube was cut from the center of one side of the steel hexagon frame to provide an opening that could pass around a potted tree trunk. On one side of

the remaining section a 1-inch nut was welded to the tube. On the opposite end of the cut section a 1-inch hole was drilled through the junction with the adjoining section. The hole was in line with the nut so that a 1-inch threaded rod could be passed through the cut section and when threaded into the nut complete a closed hexagonal ring sufficiently strong to support weights in excess of 200 kg (Figure 4.1). A 6-mm hole was drilled through the gusset at each joint for the attachment of screw bolts. The alignment of the eyebolts conformed exactly to the lifting points located along the perimeter of the upper lip of the PIP inner pots so that a circle drawn through the six screw eyes had a diameter equal to that of the upper rim of the PIP inner pot.

A small hand operated winch was mounted onto the gusset corresponding with joint E. A series of swiveling pulleys with S-hooks were laced onto 6-mm nylon cord to operate as a uniform lifting mechanism capable of lifting 150 kgs of tree, pot and soil. The end of cord coming from the winch spool was first lowered straight down to the first pulley/hook that was used for lifting the pot. The line then returned directly up to the tripod ring and passed though a single pulley attached to eyebolt E directly below the winch. The line then passed through a pulley attached to the next eyebolt D in counterclockwise order and continued to the next eyebolt C where it passed through a pulley, proceeded down to a pulley/hook and back again to another pulley on the same eyebolt. That arrangement was repeated on the next two eyebolts B and A, with the exception that the rope was finally tied to eyebolt-A.

Metal flanges were welded to the outside of the tripod ring at the midpoints between junctions A and B, C and D, and E and F and served as attachment points for three adjustable tripod legs. The legs articulated in a plane perpendicular to the attachment point on the tripod ring and allowed for easy adjustment of the tripod on uneven terrain so that that the upper ring could be maintained at a near level position.

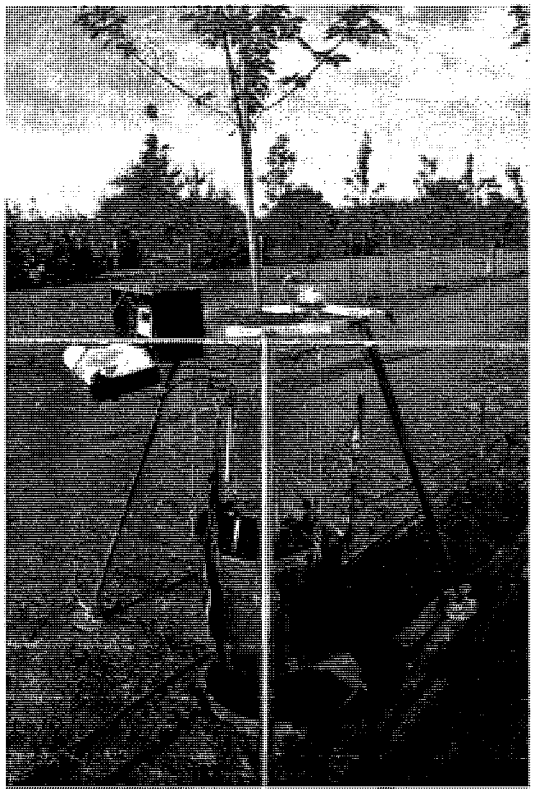
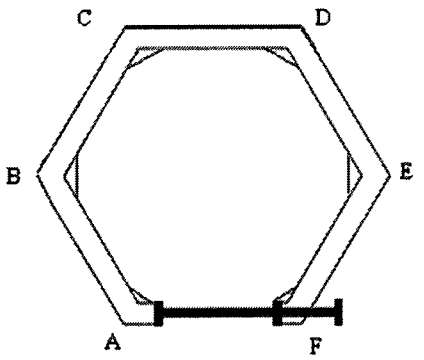


Figure 4.1. Demonstration of the open ring tripod as a scale for weighing tree lysimeters. The winch positioned toward the rear is in the locked position. The threaded rod, view right, is in the closed position to provide stability while lifting and weighing the tree. The dangling straps are used to connect the load cells to the lysimeter before releasing the weight.

The schematic shows a planar view of the tripod ring with each joint labeled A-F.



The three eyebolts B, D and F provide attachment points for three Sentran ZB1-75 S beam load cells used for weighing. These load cells were fitted with eyebolts above and below such that the upper load cell eyebolt was attached to a tripod frame eyebolt with a simple S-hook. The lower load cell eyebolt provided the attachment point for an

adjustable strap and S-hook that was used to connect the load cell to one of the three alternate lifting points.

After the inner pot was winched above the outer pot the three load cells were attached to the three free inner pot lifting points and tightened to eliminate any drop when the winch was released. Releasing the winch tension and freeing the lifting hooks put all of the weight on the load cells that were connected to a Sentran NB3 summing box and Sentran KB3 digital indicator powered by a 12 volt battery. This configuration allowed the weight to be read directly. The components were mounted into a modified tool box such that it could be easily attached to the tripod frame and removed when not in use. The system was accurate to .02% of applied load.

Tripod Operation and Pot Irrigation

The tripod leg length was adjusted to a height just below the lowest branch on the potted trees. With the threaded rod in the open position, the tripod was pivoted on one leg around the trunk of the tree and quickly adjusted so that the upper ring was fairly level and the trunk was approximately centered in the ring. The threaded rod was then screwed a few turns into the receiving nut closing the tripod ring. The winch hooks were attached to the plywood base and the inner pot was lifted. Because of minor friction along the series of pulleys and variations in the pot/tree center of gravity the trunk was guided by hand as the pot was lifted. Once free of the outer pot the inner pot and tree would shift slightly depending on weight distribution. The trunk was then manipulated so that the upper lip of the lysimeter pot was close to level and the trunk did not touch the tripod ring. The winch was then set in the locked position and the load cells were attached and

adjusted to support the tree in a near vertical position. Once attached, the winch was released so that the entire load was distributed to the load cells and the weight was recorded. The weight of the lysimeter pot was then returned to the winch, the load cells disconnected and the lysimeters pot lowered back into position inside the outer pot. The threaded rod was unscrewed, the tripod was pivoted away from the tree and the unit was lifted and carried to the next tree. A single researcher can carry this tripod quite easily. This system eliminates the need for double handling of the lysimeters or the awkward manipulation of trees too tall for a conventional tripod.

Prior to initiation of a drydown cycle, the lysimeters pots would be saturated the evening before the start of measurements. This allowed sufficient time for complete soil saturation and drainage of excess water. A control lysimeter, constructed identically to other lysimeters but without a planted tree, was weighed to estimate soil surface evaporation during the first days of a drydown cycle. Subsequent days produced a dry soil crust on the surface of lysimeters constructed in 2005 with Freeman maples using composted potting media. This barrier to further evaporation eliminated the need for adjustments to ET. The soil surface of lysimeters constructed in 2006 with green ash using clay soil was covered with white opaque plastic so that evaporative soil water loss was greatly reduced and therefore, an insignificant portion of tree ET. During the drydown periods soil moisture was measured using a portable Trace time domain reflectrometry probe.

System Error

Matching the dimensions of the tripod ring to the inner pot was critical because the error in weight measurements is partially composed of increased tensions calculated as the cosine of the angle from vertical for each of the load cells. Although the trees were planted in as near a center position as possible, an off center position and irregularities in canopy and root development shifted the lysimeter center of gravity. Once the lysimeter pot was lifted clear of the outer pot, the three point configuration of the load cells would shift away from vertical as a function of the final balance point and any deviation of the pot surface and tripod ring from horizontal. As long as the tripod ring was visually level and the trunk of the tree was kept within the center of the tripod ring, the angle of deviation from vertical was less than two degrees. That calculated to less than 0.06% of applied load. This error combined with the load cell accuracy of 0.02% of applied load produced a weighing accuracy of ± 80 grams for a 100 kg load. This maximum error was sufficient to accurately determine ET. Under windy conditions, variable loading of the load cells caused fluctuation in the readout. However, hand stabilization of the tree was sufficient to reduce that fluctuation and produce stable readings.

4.3) Results and Discussion

Freeman Maples

During September 2003, six Freeman maple lysimeters were weighed to determine water use as part of a series of drying cycles designed to better understand the effect of periodic and repeated drought stress on ET. Although September is well into the beginning of

dormancy for many species, Freeman maples continued active transpiration and leaf growth.

For each day a reference ET_o was calculated for a bluegrass turf (Allen et.al.1998). Daily water use in the maples was nearly identical to ET_o (Figure 4.2). This correlation ($R^2 = 0.94$) existed despite the continued drop in soil moisture during the same six day period. On the morning of September 25, 2003, when the potting soil moisture dropped below five percent, sunrise caused immediate leaf wilting followed closely by leaf tip burn. That observation triggered irrigation of the trees to avoid permanent leaf damage.

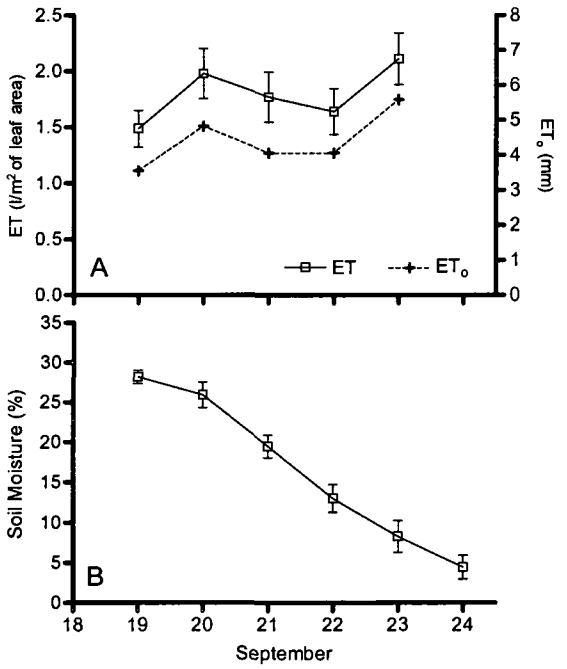


Figure 4.2 Consistent ET despite soil moisture depletion in Freeman maple lysimeters during 2003. Daily ET (A) for each of six Freeman maple lysimeters is measured in liters per square meter of leaf area. Average canopy size was 1.78 for the six trees measured. Lysimeters were located randomly in two rows of 25 trees each arranged within a northern and southern array. ET_o is reference ET for a grass as calculated using the Penman-Monteith equation. Volumetric soil moisture (B) was measured using a portable Trace TDR probe. Error bars represent one standard deviation.

The abrupt change from the unrestricted ET to drought stress induced leaf damage was caused by the water holding properties of the potting medium. The Organix®media held

water with a matric potential between –0.10 and approximately –0.20 MPa. In the saturated #20 pot this equated to between 13.6 to 17.7 liters of available water. Freeman maples under drought stress exhibited stomatal control to regulate ET (Zwack et.al., 1998 and 1999; Bauerle et.al., 2002). However, even during a daily depletion of water in the potting medium, enough remaining water was available to allow tree ET to reflect atmospheric demand. When that water was finally depleted, there was a rapid decline in leaf water content that could not be controlled by stomatal regulation.

In 2004, four trees, maintained in the same #20 pots a second year, were measured for water use during a period of peak summer heat. Average canopy leaf area, just prior to leaf drop in October of the same year, for the selected trees was 5.29 m^2. The 2004 canopies were significantly larger than in 2003, and increased water use.

In 2004, as in 2003, daily tree ET followed the pattern of calculated ET_o of a reference grass at soil moisture greater than five percent (Figure 4.3). When soil moisture dropped below five percent, on August 6, 13 and 14, 2004 there was an immediate decline in water use to an amount far less than would be predicted based on the reference grass ET_o. This decline in tree ET was followed immediately by leaf wilt and leaf tip burn. During the 2004 drying cycles, but prior to drought stress, Ψ_{pd} was consistently –0.07 MPa. When soil moisture dropped below five percent, Ψ_{pd} dropped to between –0.12 and –0.27 MPa. This drop is minor compared to Ψ_l in other studies (Zwack et.al. 1999). However, the lower Ψ_{pd} does indicate the initiation of drought stress. At the onset of drought stress

the potential for rapid and permanent leaf damage, observed in 2003, increased and the trees were irrigated.

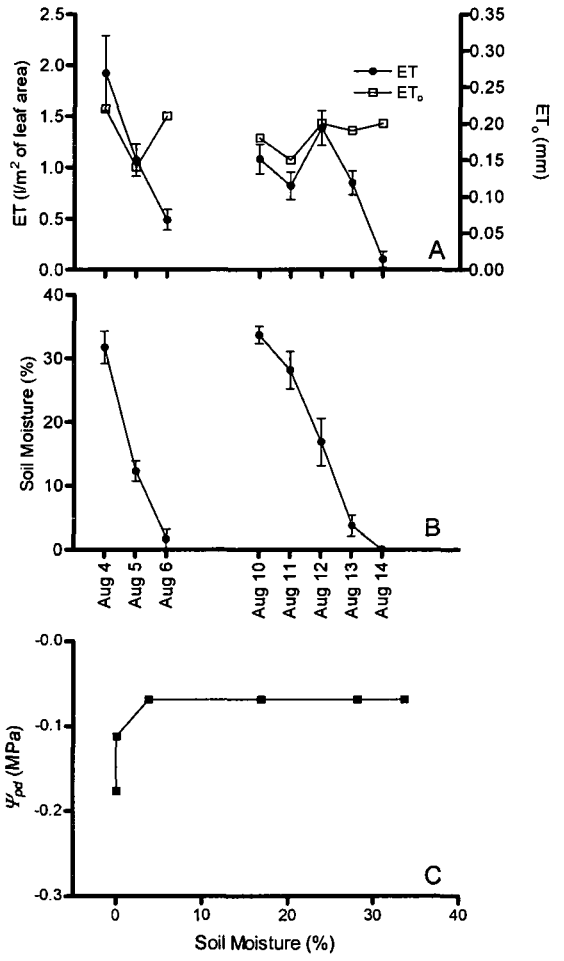


Figure 4.3 Effects of soil moisture depletion on ET and Ψ_{pd} in Freeman maples during 2004. Figure 4.3a presents ET in liters/m^{-2} of leaf area for four tree lysimeters between August 4 and 14, 2004. ET_o is reference ET for a grass as calculated using the Penman-Monteith equation. Deviation from that expected pattern occurred on Aug 6, 13, and 14, 2004 when potting soil moisture dropped below 5 percent (B). The soil moisture characteristics curve (C) shows the lack of drought stress until soil moisture dropped below five percent. Error bars represent one standard deviation.

Green Ash

Between September 2 and 8, 2006 four green ash lysimeters were used to determine the limits of drought stress and the effects of that extreme stress on A_n and g_s. Lysimeter ET on September 2, 3, 4 and 5 was directly coupled to atmospheric demand as represented by the reference grass ET_o (Figure 4.4). Average soil moisture for the same period

dropped from 39 to 24 percent. On September 6, 2006 a 62 percent drop in daily water use corresponded with a 6.4 percent drop in soil moisture and a drop in average Ψ_{pd} from –0.47 to –2.1 MPa. The drop in average soil moisture to 18 percent represented a threshold that induced the onset of drought stress. On September 7, 2006 there were further declines in tree ET and Ψ_{pd} that corresponded with increasingly dry soils despite a constant ET_o demand. Tree ET continued at a reduced level through September 7 causing further reductions in soil moisture. The continued soil moisture depletion caused Ψ_{pd} on September 8 to drop to a low of –5.28 MPa. At that point leaf tip burn was observed and the lysimeters were irrigated.

During the period of stress between September 5 and 7, 2006 A_n dropped dramatically during mid-day (Figure 4.5). That mid-day decline rebounded the following morning but to a progressively lower level. With lower morning VPD levels on September 6 and 7, 2006 A_n was still measureable at rates greater than the previous day's mid-day measurements. However, by the morning of September 7, 2006, A_n was only as high as mid-day measurements on September 5, 2006. The morning A_n was possible due to g_s rates that exceeded the depressed mid-day values on September 5 and 6, 2006. The A_n /g_s relationship showed a strong correlation (R^2 = .81) with A_n peaking at g_s rates just below 100 mmol $m^{-2}s^{-1}$ (Figure 4.6). The progressive loss of water through continued low rates of g_s rapidly reached terminal levels in the confined pot system and did not represent the more gradual decline in established trees (Chapter 1).

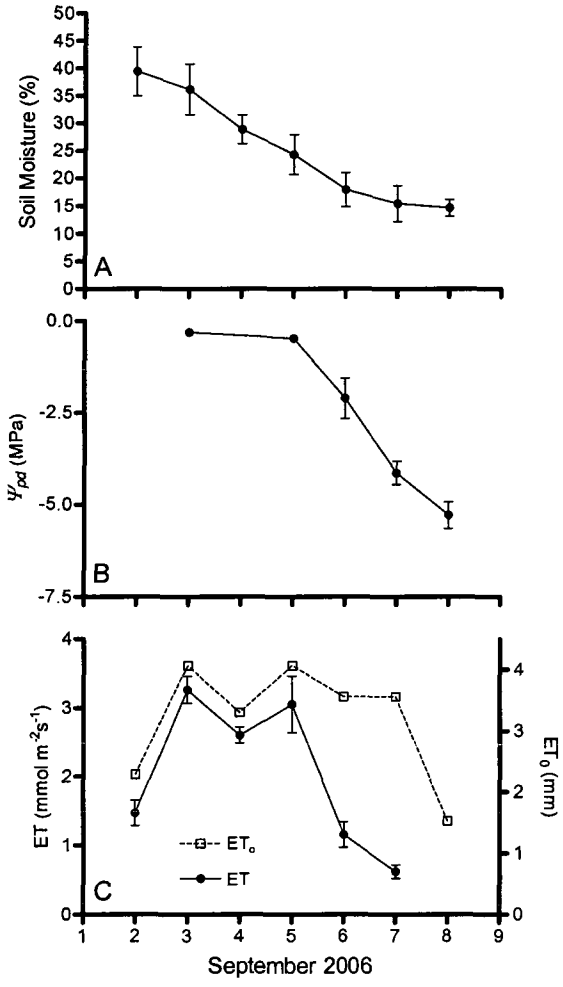


Figure 4.4 Effects of declining soil moisture on Ψ_{pd} and ET in four green ash lysimeters. Graph A shows the steady decline in soil moisture during the drydown period from September 2 through September 8 for four lysimeters. The drop in predawn leaf water potential on September 6 (B) corresponds with average soil moisture of 18 percent and a dramatic drop in ET (C). ET_o indicates that the reduction in ET was not associated with reduced evaporative demand. Error bars represent one standard deviation.

The data collected on the potted green ash trees were used to support the larger study of established trees where drought was not able to reduce Ψ_{pd} of established trees to -3.14 MPa. These lysimeters show that Ψ_{pd} may reach levels as low as –5.28 MPa before permanent leaf damage occurs. While these values are similar to European ash, that reached Ψ_{pd} of -6.0 MPa while maintaining g_s (Guicherd et al. 1997), minimum Ψ_{pd} of established green ash under severe drought was -3.14 MPa. This indicates that the

drought conditions necessary to cause acute symptoms of drought stress are rarely experienced in urban landscapes.

The benefit of extreme drought tolerance to green ash is unclear. Severe drought resistance is not an adaptation that would be expected from the native range of the species. Rather it may fall within the boundaries of metabolic processes inherent in most trees that have evolved in concert within an environment which, as a whole, resists the drought conditions imposed in this study. The introduction of green ash into controlled urban environments extends the range of the species but only under rare conditions would cities capitalize on the demonstrated drought resistance. Although green ash will withstand extreme drought conditions, continued growth of the urban canopy and associated carbon sequestration requires sufficient irrigation to optimize g_s.

4.4) Summary

The available water storage capacity of Organix® composted potting media had unique water storage characteristics quite different from soil. The potting media provided available water from saturation to five percent moisture by volume. As the stored water was depleted through ET, the media become critically dry before drought stress was induced in the potted trees. The greater availability of water delayed g_s regulation and allowed rapid growth associated with maximum ET to continue until the abrupt onset of critical drought stress. These results have direct applicability to tree nurseries growing trees in pots. Nursery managers using similar media while attempting to optimize growth and conserve water must be cautious and avoid crossing that drought stress threshold.

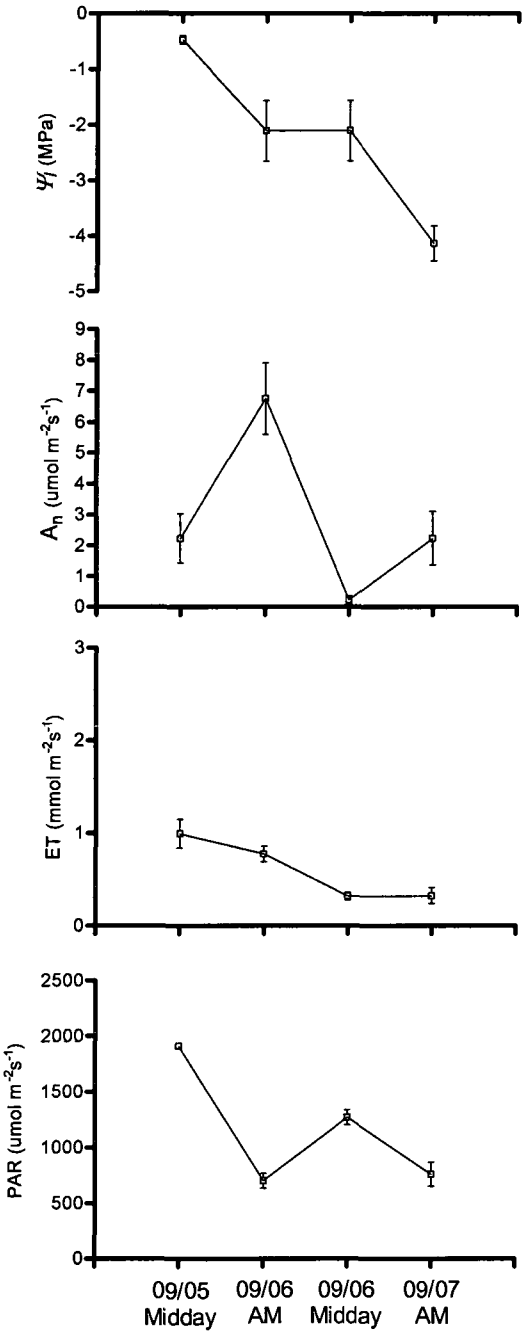


Figure 4.5 Effect of drought stress on Ψ_l, A_n, and ET on four green ash lysimeters. PAR, ET and A, measurements were collected from leaves immediately removed from the tree and measured for Ψ_l. The rapid reduction in Ψ_l is an artifact of the confined pot system with limited soil water storage. As ET rates dropped, A_n became progressively more depressed with only minor recovery during the cooler mornings. Error bars represent one standard deviation.

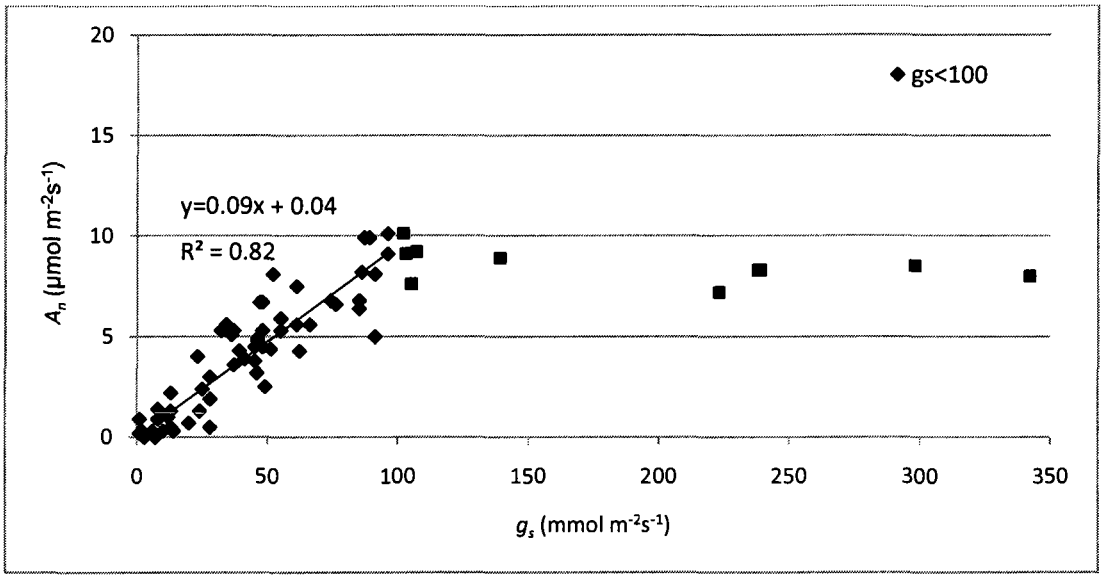


Figure 4.6 Relationship of g_s and A_n during progressive drought stress in four green ash lysimeters. The regression line represents all A_n measurements on four trees, for the entire drying period between September 2 and September 8, with gs lower than 100 mmol $m^{-2}s^{-1}$. High g_s reflected fully hydrated conditions that allowed for increased ET without increased A_n.

Green ash lysimeters constructed of native clay soils were used to create an environment that closely reflected established trees in the landscape. As soil moisture levels declined in these confined systems, g_s regulation and corresponding depression of A_n was similar to established trees undergoing drought stress. As soil moisture declined further and extreme drought stress was induced, g_s and A_n also decreased but were still measureable at a Ψ_{pd} of -5.28 MPa. Below -5.28 MPa permanent leaf damage occurred. Considering the range of drought stress tolerated by green ash and the unlikelihood of those conditions occurring in a managed landscape, negative effects of seasonal drought are minimized in green ash.

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Appendix A

Irrigation and Tree Growth 1996 -2005

In 1996, treatment blocks were randomly chosen to receive one of three irrigation levels. Blocks 1, 4, and 8 were scheduled to receive irrigation at the rate of 160 percent of evapotranspiration (ET_o) as calculated for bluegrass of average quality (Allen et al. 1998). Blocks 3, 6, 7 were scheduled to receive 80 percent of ET_o and Blocks 2, 5, and 9 were scheduled to receive 40 percent of ET_o (Figure B-1).

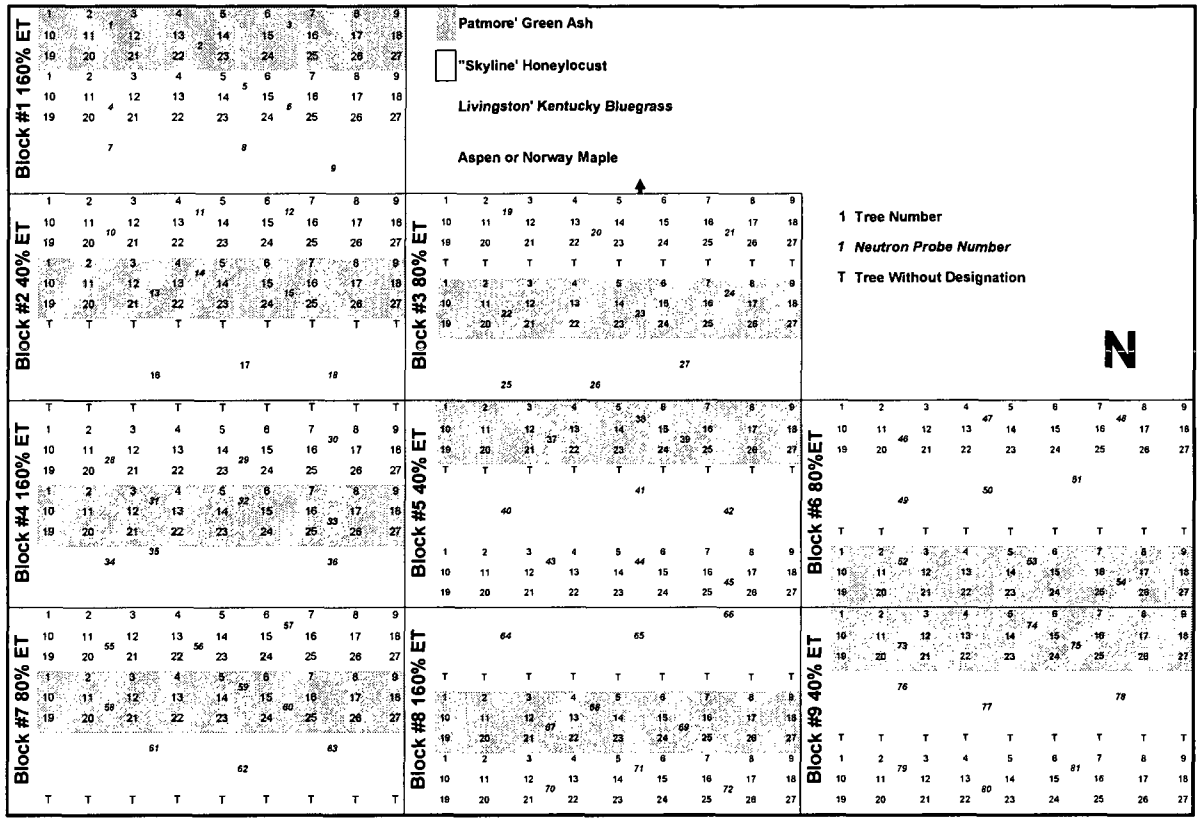

Figure A-1 Planting diagram of the Agriculture Research Development and Education Center (ARDEC) Tree and Turf Research Site. Percent ET designation represents the amount of irrigation received from 1996 through 2004 based on calculated ET bluegrass turf. Windbreaks to the north, west and east are not shown.

An anecdotal review of the actual irrigation applied indicates that these schedules were not always maintained in years prior to 2004. Research assistants would add additional irrigation based on the appearance of turf at the site. In addition, careful monitoring of

actual ET_o was often abandoned for a fixed weekly watering schedule. Divergence from the treatment regimes was confirmed by reviewing soil moisture data.

Soil moisture data for 2000, 2001 and 2003 were similar for Block 8 (160 percent ET_o) and Block 9 (40 percent ET_o) (Figure B-2). The data points are averages from three nuclear density gauge access tubes on the specific dates presented. In both blocks, soil moisture data are higher than measurements collected in 2005 at all depths. Analysis of all data for all treatment blocks from all three years prior to 2005 shows that soil moisture in all blocks except Block 1 were statistically identical. That similarity dismisses the idea that increasing tree size contributed to greater soil water depletion in 2005. If that were so, there would be a trend in decreasing soil water content for years 2000, 2001 and 2003. In addition, data for years 2000 and 2001 are not statistically different between the different treatments despite Block 8 scheduled to receive four times the water. Considering the uniformity of soil moisture in Blocks 2 through 9 regardless of irrigation treatment, it is apparent that treatments were irrigated at a greater rate than intended during the years prior to 2005.

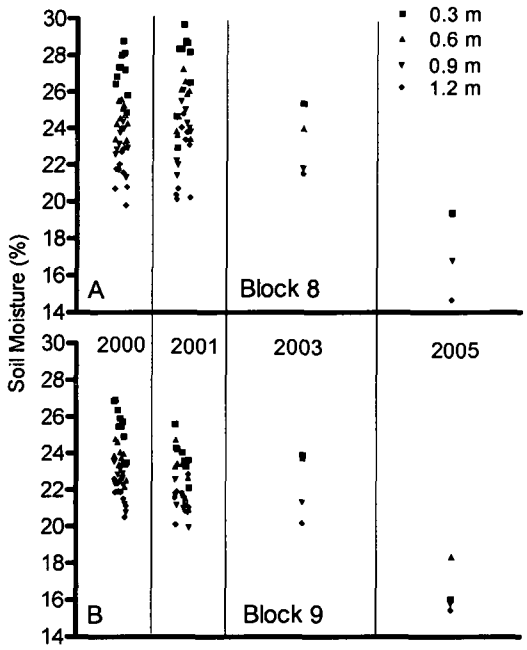

Figure A-2 Similarities in Block 8 (A) and Block 9 (B) depth specific soil moisture measurements for years 2000, 2001, 2003, 2005 . Data points represent average soil moisture from three access tubes in each block at four depths below ground surface (0.3, 0.6, 0.9 and 1.2 meters). When comparing specific depths, all measurements in 2000, 2001 and 2003 are significantly greater than measurements in 2005. Measurements for the different treatments within the years 2000, 2001 are not significantly different.

Pooled trunk diameter data from all three blocks within each treatment show a statistical difference between treatments only because Block 1 skewed the data for the 160 percent irrigation treatment. To avoid edge effects only the seven interior trees in the center row were used to calculate the analysis of variance. Trunk diameters for individual treatments show that trees in Block 1 stand apart from the rest of the blocks (Figure B-3). Analysis of only Blocks 2 through 9 shows that tree diameters were not affected by irrigation treatments.

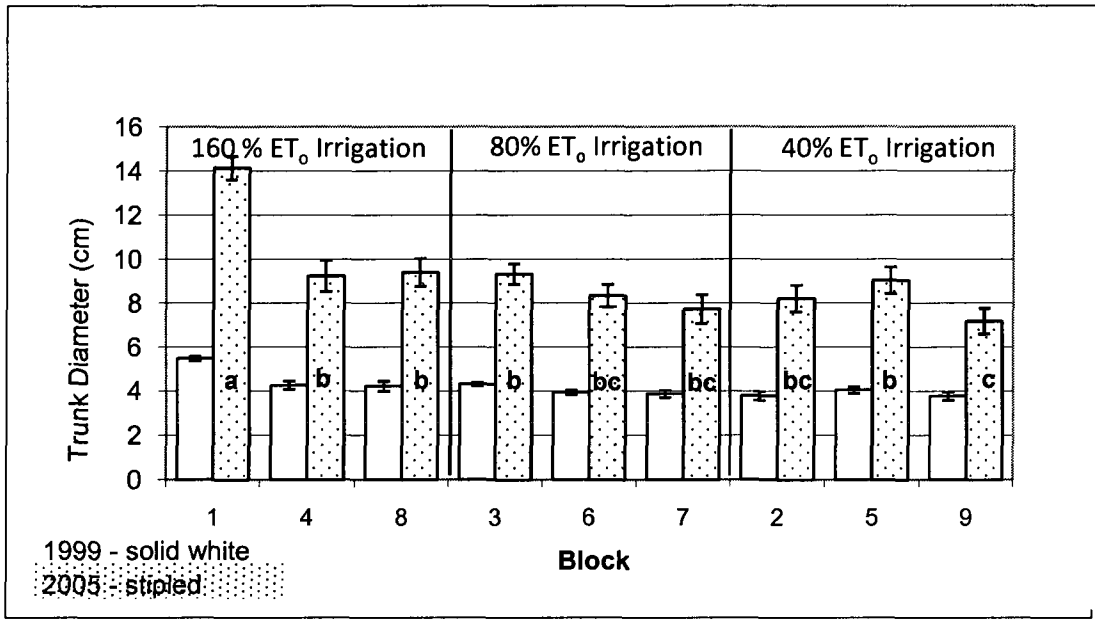

Figure A-3 Effect of three irrigation treatments on 1999 and 2005 Tree trunk diameters of green ash trees at ARDEC. ET_o is for bluegrass using the Penman Monteith equation. Each column is the mean of seven interior trees. Error bars represent the standard error for each block. Block 1 received additional water not controlled by irrigation. Trees with the same letter in 2005 are not significantly different.

Appendix B

Nonstructural Carbohydrate Analytical Protocol

(Precautionary Note: This protocol utilizes concentrated acids and bases. Wear protective goggles, gloves and apron. Follow all safe lab procedures. Acids and bases are added to water and other solutions. Do not use bottles or restricted opening vessels when adding acids and bases to water. Properly store all wastes.)

Reagents

Anthrone:

0.2g anthrone in 100ml 72% Sulfuric Acid
Prepared daily – let stand for 30-40 minutes with occasional shaking until it is perfectly clear

o-dianisidine dihydrochloride:

Mix 50 mg of *o*-dianisidine dihydrochloride (Sigma D-3252) in 20 ml of nanopure water

Glucose Oxidase/Peroxidase (PGO):

Dissolve one capsule of PGO enzymes (Sigma P-7119) with 100 ml of nanopure water in an amber bottle. Add 1.6 mil of *o*-dianisidine diydrochloride solution. Remove gas from solution by sonication and vacuum for 30 minutes

30% Sodium Hydroxide

Combine 6mL of 50% NaOH solution into 4ml of nanopure water

0.1 N Acetic Acid

In an open beaker, add 0.575mL of glacial acetic acid to 95mL of nanopure water
Mix well and bring to volume of 100mL with nanopure water

.05 M Sodium Acetate Buffer

Add 0.284mL of glacial acetic acid to 90mL of nanopure water
Adjust to pH of 5.1 with addition of 30% sodium hydroxide
Bring to volume of 100ml with nanopure water
(alternatively, prepare from stock sodium acetate salt)

80% Ethanol:

Bring 800mL of 100% ethanol to a volume of 1L with nanopure water

α- amalaze from *Bacillus licheniformis* (Sigma A3403)

Dilute 10 fold in 0.05M sodium acetate buffer adjusted to pH 7.2

AMG - Amyloglucosidase from *Aspergillus niger* (Sigma A3042)

Dilute 50 fold in 0.05M sodium acetate buffer adjusted to a pH of 4.5

1-(Trimethysilyl)imidazole – Pyridine mixture (TriSil) (Sigma 92718)

Hexane

Standards

Glucose, Fructose, Sucrose, Raffinose

For sugar analysis by anthrone: 650mg/L of selected sugar diluted in 80% ethanol to bracket sample concentrations

For carbohydrate ratio tests use 10 ug of selected sugars individually and combined

For starch analysis by PGO: 25 – 200 mg/L of glucose in 0.05M sodium acetate brackets the reaction range

Starch

Starch standards should be prepared by weighing between 1 and 10 mg of potato starch and handling as samples.

scyllo-Inositol (Sigma I8132) – 10ug used in combination with selected sugars

Blanks

80% Ethanol

Blank started at step 3 in sugar analysis

Nanopure water

Blank and replicate blank started at step 2 in starch analysis does not include addition of enzymes

α- amalaze and AMG

Multiple blanks started at step 2 in starch analysis to determine the contribution of glucose from the enzymes.

Analytical Methods

Sugar Extraction and Analysis

1) Grind each sample to pass through #40 sieve
2) Weigh 25mg of sample into a 15ml test tube.
3) Add 1 or 2 boiling chips
4) Add 2ml of 80% Ethanol
5) Place test tube in 95 ^{0}C water bath with marble cap for 10 minutes
6) Centrifuge for 5 minutes at 3000 rpm
7) Aspirate supernatants and save

8) Repeat steps 2 through 5 two more times
9) Bring to standard volume of 10mL with 80% ethanol
10) Dry sample residual and store in freezer for starch analysis

Anthrone Reaction

1) Turn on water bath and bring to boil.
2) Prepare a bed of ice for rapid cooling.
3) In appropriate microplate well combine
   25 ul of sample solution or standard solution
   225 ul of anthrone solution to each well
4) Mix by pipetting
5) Cover microplate with adhesive seal
6) Place in steam of boiling water bath for 12 min and then immediately place onto bed of ice until microplate is cold and the reaction has stopped.
7) Dry microplate and read at 630 nm
8) Determine confidence value (CV)
   Standard Deviation /Average * 100
9) Repeat or dilute as necessary

Test for Relative Abundance of Carbohydrates

1) Prepare standards using 10 ug of selected sugars.
2) Dry down enough sample to produce carbohydrate concentrations similar in magnitude to the standards.
3) React 50 ul of TriSil in closed glass tube at 80 oC for 20 minutes. This should replace active hydrogen atoms with trimethylsilyl groups and make the sugars volatile.
4) Blow down solution using nitrogen or inert gas
5) Add 100ul hexane. This will allow the sugars to move into solution without putting the rest of the precipitate in solution.
6) Pipette the solution free of residual precipitate and store at room temperature.
7) Prepare sample vials for GC/MS and run.
8) Determine relative abundance of complex sugars, sucrose and raffinose by measuring the area of specific peaks and setting them to standard concentrations.
9) Determine glucose equivalent using the estimated percentage of sugars
   For $X = 0$ to 2.5

$$\begin{aligned}\text{Sugar}_{(\text{as glucose})} &= (1 - (P_s + P_r))(\text{mXg} + \text{bg}) \\ &+ P_s(342.30/180.16)(2.41y - 0.01) \\ &+ P_r(594.52/180.16)(3.61y - 0.14)\end{aligned}$$

Where: P_s = Estimated percent of sucrose/100
P_r = Estimated percent of raffinose/100

10) Calculate glucose concentration of sample

$$y_g = a + bx$$

a = intercept
b = slope
x = absorbance units at 630nm
y_g = mg of glucose/10ml

$$y = \frac{y_g v}{d_w}$$

y = mg of glucose / mg of sample
v = original volume of extract = 10 ml
d_w = original dry weight of the sample

Starch Extraction and Analysis

1) Place residual starch sample in 15mL test tube
2) Add 2mL of nanopure water and suspend residue
3) Place tubes with marble cap in a boiling water bath for 45 minutes to gelatinize starch
4) Cool tubes in ice water
5) Prepare dilute mixtures of acetic acid and sodium hydroxide for pH adjustment
6) Adjust pH to between 6.6 and 7.5 with the addition of acetic acid or sodium hydroxide
7) Add 200 mL of α- amalaze preparation and cap
8) Mix by inversion or vibration
9) Incubate in 80 - 85 °C water bath for 30 minutes
   Mix frequently for 3 minutes and occasionally for final 27 minutes
10) Cool to room temperature
11) Lower pH to 5.0 ± 0.1 with acetic acid
12) Add 1 ml of AMG preparation and cap
12) Mix by inversion or vibration
14) Incubate in 50 - 55 °C water bath with shaking for 60 minutes
15) Bring to standard volume of 5mL with nanopure water
16) Centrifuge at 3000 rpm for 5 minutes
17) Aspirate supernatant
18) Bring samples to 10 ml volume with nanopure water
   Additional dilutions may be needed to bring sample into range of reaction

PGO Reaction

1) In appropriate microplate well combine
   20 ul of sample
   200 ul of PGO reactant
   Pipette carefully to eliminate any bubbles (do not completely expel reactant)
2) Cover microplate with plastic lid and wrap in aluminum foil
3) Place on shaker for 45 minutes
4) Read at 450 nm

5) Determine confidence value (CV)
   Standard Deviation /Average * 100
6) Repeat or dilute as necessary

7) Calculate glucose concentration

$$y_g = a + bx$$

a = intercept
b = slope
x = absorbance units at 630nm
y_g = mg of glucose/100ml

$$y = \frac{y_g d_f h_f v}{d_w}$$

y = mg of glucose / mg of sample
d_f = dilution factor = 10
v = original volume of extract = 10 ml
d_w = original dry weight of the sample
hf = starch hydrolosis factor = 0.9